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PHYSIOLOGY OF DEVELOPMENT OF THE FEATHER

V. EXPERIMENTAL MORPHOGENESIS[1]

(Five text figures; eight plates)

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TABLE OF CONTENTS

PAGE
I. Introduction 104
II. The normal breast and saddle feather: anatomy and development of the rhachis . 104
  1. Anatomy of the normal breast feather 104
  2. The development of the rhachis 106
III. The structure and functions of the papilla 106
IV. Experiments on the papilla 109
  1. Technique 109
  2. Introduction to experiments 110
  3. Transplantation experiments: orientation and tract specificity of papillae . . 111
  4. The defect experiments 112
    A. Introduction 112
    B. Classification and description of abnormalities 113
    C. Relation between types of operation and results 115
  5. The isolation experiments 117
  6. Recombination experiments: chimaerae 118
  7. Some special experiments 119
  8. Later generations of feathers from operated papillae 120
  9. The occurrence of abnormalities in natural feathers 122
  10. Summary of results 122
V. Theoretical 123
  1. Introduction 123
  2. The elementary abnormalities 123
    A. The rhachis 123
    B. The barbs 124
    C. The theory of reversed barbs 126
  3. The theory of tangential movements 129
  4. The organization of the papilla 130

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I. INTRODUCTION

ALTHOUGH a great many studies have been presented on the physiology of development of feathers, none have dealt with the primary mechanism of development by methods of isolation of parts, their transplantation and recombination, such as are familiar in experimental embryology. Our own previous attempts to operate in such ways on the embryonic ring of cells known as the "collar" failed completely to yield positive results.[2] But, when we finally decided to operate directly on the papilla, which remains behind at the bottom of the follicle when the feather is plucked or naturally dropped in the process of molting, the first attempts yielded significant results, which were then extended by systematic experiments.

The operations have yielded a great number of abnormalities different from any hitherto recorded, and the types of abnormalities are definitely related to the types of operations. We are thus furnished with new material for a theory of development of feathers. As in experimental embryology, twins, "chimaerae," and many significant abnormalities and defects contribute to the theory.

Feathers of the breast tract of Brown Leghorn and White Leghorn fowl were used chiefly, but feathers from other tracts, especially from the saddle tract, were used occasionally for comparison and for special purposes such as the production of chimaera feathers.

II. THE NORMAL BREAST FEATHER: ANATOMY AND DEVELOPMENT OF THE RHACHIS

As most of the experiments deal with feathers of the breast and saddle tracts, photographs of representative feathers from these tracts are presented in Plate I (Fig. 6, *A* and *B*) as a basis for reference. It is also necessary to emphasize certain features both of the anatomy and of the development that have not hitherto been described or are not generally understood, in order to present the results of the experiments. These data are limited to the feathers of the breast tract.

1. ANATOMY OF THE NORMAL BREAST FEATHER

The feather is a difficult subject for dissection, and the incentive to tackle it, which we acquired from our experiments, has been lacking. Dissection of the feather requires a special technique, for the parts are so firmly bound together by tough keratin that dissecting instruments are otherwise of no avail. The method that we employed was immersion in 5 per cent KOH in aqueous solution; but this will not penetrate unless the external keratin is opened to permit access of KOH to the medulla of the rhachis. In order to do the least possible injury, a longitudinal incision is made along the ventral surface of the rhachis in the fluffy region; after this the feather is immersed in the KOH solution by a weight and allowed to remain in it from 8 to 12 hours. It is then washed in water and preserved in 70 per cent alcohol. The alkali passes along the entire length of the rhachis by diffusion through the medullary cells and softens the connections so that dissection becomes easy.

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[2] Attempts to operate directly on the collar of regenerating feathers *in situ* usually resulted in death of the feather but in any case yielded no positive results. Dr. Graham P. DuShane with our collaboration then made a very extensive series of transplants of portions of the collar to the chorio-allantoic membrane; these often yielded growth, but none of them contained any formed elements of the feather. The most conspicuous products were long fibers, presumably of keratin.

The feather to be dissected is placed in a large Petri dish flooded with 70 per cent alcohol. It is held at its apex by forceps while another pair of fine forceps in the operator's right hand gets a firm grip on some four or five barbs of one side of the vane close to their attachment to the rhachis and at a distance of about 1 cm. from the apex of the feather. With the feather thus caught in the two pairs of forceps, the barbs may be separated from the central part of the rhachis in a continuous strip by a gentle, steady movement. The forceps are moved back to new positions each time that a length covering some thirty or more barbs is detached. This can be continued to the calamus if so desired. The other side is similarly stripped off, leaving behind a relatively thick central rhachis with only a small tuft of barbs at its apex (Pl. II, Fig. 8).

Lillie and Juhn (1938) had concluded that the axis of the shaft is of entirely different embryological origin from lateral components formed from the bases of the barbs; but the anatomical consequences were not studied. The potash treatment makes this possible. Further dissection shows that the lateral, or ramic, components are composed exclusively of special modifications of the bases of the barbs, which we call the *barb petioles* (Pl. II, Fig. 9).

Each barb petiole (Pl. II, Fig. 10, *p.*) is a flattened plate curved to conform to the central rhachis; it forms a slight angle with the free axis of the barb and carries an extension of the series of proximal barbules (*p.r.*), about twelve to fourteen in number in the vane of a breast feather, as far as the insertion of the next proximal barb; these barbules, which appear to arise from the shaft between the insertions of barbs, thus belong primarily to the latter. Long keratin fibers (*f.*) arising within the petioles extend a distance of two or more barb intervals, thus building up a continuous fibrous base of the lateral components of the rhachis.

The medullary cells of the barbs terminate above the bases of the petioles (Pl. II, Fig. 10, arrow) and are thus not continuous with the medullary cells of the central rhachis. It is an outstanding feature of the petioles forming the ramic components of the rhachis that they are entirely devoid of medullary cells.

However, at the very apex of the feather the medullary cells of the two apical barbs, one on each side, are continuous with one another and with those of the rhachis. The same relation may be found in the second barb on each side, though the connection is constricted; the connection is lost in the third barb, though we do not assert that this is exactly so in all specimens. It is about in this region, then, that we place the apical termination of the central rhachis, the medullary cells of which are continuous with those of the first two barbs on each side. This anatomical determination is confirmed by study of the development.

The central rhachis itself shows evidence of bilateral structure in the ventral groove and the heart-shaped form of the medulla in cross-section; quite frequently in our experimental feathers, and once in a natural breast feather, we have observed complete lateral doubling of the medullary cells involving part or all of the length of the rhachis. The central rhachis arises embryologically from a spreading base in the collar extended laterally, and the disposition and orientation of the melanophores in the growing base indicate a bilateral origin for those which enter the rhachis (cf. Lillie and Juhn [1938], p. 440 and Figs. 7 and 17). Finally, we have numerous (twenty-one) cases in the experiments recorded in this paper in which only one lateral half of the rhachis is formed. We therefore regard the rhachis as composed of two lateral halves, in each of which we recognize a central component and a lateral (ramic) component.

2. THE DEVELOPMENT OF THE RHACHIS

The rhachis does not begin to arise until after a large number of apical barbs have formed. On the sixth day of regeneration about fifty of approximately equal length are found in the dorsal part of the germ as a continuous series occupying about two-thirds of the circumference. Their orientation is strictly axial, and they accordingly lie parallel to one another and to the long axis of the cylinder (Fig. 1, *a;* cf. Lillie and Juhn, 1932). They are then added to from both sides of the ventral field progressively and very rapidly toward its center by new barb apexes until most of the field is occupied. During the sixth day tangential movements of the barbs are absent.

After the dorsal barbs have reached a length of about 2 mm. (on or about the seventh day), two to four of them in the center of the dorsal surface fuse at their bases, and the central rhachis arises in the collar immediately beneath them, fusing with the conjoined base (Fig. 1, *b* and *c*). At this time tangential movements of the barbs begin at their junction with the collar, producing a bend toward the rhachis in the case of the bases of the original barbs; as this movement extends ventrally, the barbs begin to describe spiral courses around half of the cylinder on each side. The most ventral ones thus define in the center of the ventral field a ventral triangle from which the tangential movements are directed to the right and to the left. As the central rhachis grows axially, the series of forming barbs moving toward it become united to it by their forming petioles.

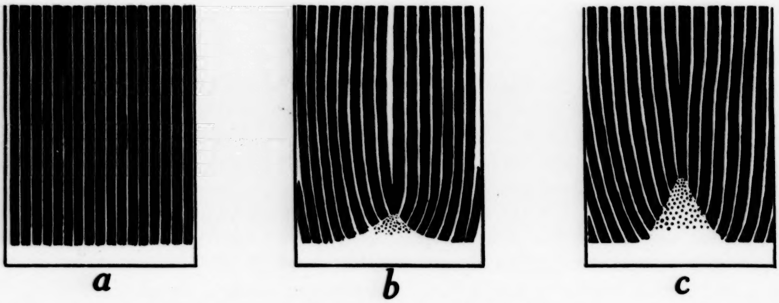

FIG. 1.—Diagrams of the bases of barbs in the center of the dorsal surface of feather germs of a Brown Leghorn capon: *a*, of 6 days' regeneration, *b*, of 7 days', *c*, of 8 days'. The origin of the central rhachis (stippled) is shown in *b* and *c*. The diagrams are based on cleared mounts of entire feather germs. The framed space below the barbs represents the margin of the collar (cf. the text).

Barb petioles constituting the lateral components of the rhachis are not formed in the case of the two apical barbs and occasionally of one or two more, which fused at their bases before the central rhachis began to form. This agrees with the anatomical facts already alluded to (p. 105). Barb petioles, and hence lateral components of the rhachis, are formed from this point proximally.

III. THE STRUCTURE AND FUNCTIONS OF THE PAPILLA

The term "papilla" has been used in diverse ways. In early studies on development of the feather no distinction was made between "papilla" and "pulp." The germ for replacement and regeneration has also sometimes been regarded as something formed anew by the corium for each occasion (Woitkewitsch, 1936). It is therefore necessary to emphasize our view that, while the pulp is formed anew for each replacement of a feather, the *papilla* is a persistent structure from the time of its embryonic origin through all successive generations of feathers.

In all our numerous experiments no follicle from which the papilla has been removed has ever regenerated a feather; and, on the other hand, an isolated papilla implanted subdermally under favorable conditions regenerates both feather and follicle (cf. p. 112).

The papilla has two main functions—first, to produce the vascular pulp concerned in the nutrition of the growing feather, which was dealt with fully in the preceding paper of this series (Lillie, 1940), and, second, the morphogenetic function, with which the present paper deals, to which reference was also made in the preceding paper (p. 145).

The relations of the papilla to the feather cylinder[3] during regeneration are shown in Figure 2 and in more detail in Figure 3. The papilla is embraced and constricted by the margin of the collar (inferior umbilicus). The distal portion above the umbilicus is continuous with the pulp; and the basal portion below the umbilicus, shaped like an inverted mushroom, is firmly anchored by its margins in the wall of the follicle. The broken line in Figure 3 represents approximately the boundary between the pulp-forming part of the papilla and the part concerned with control of morphogenesis. The papilla of a feather in process of regeneration will be called an "active papilla."

Figure 11 (Pl. III) represents an axial section of an active papilla immediately after

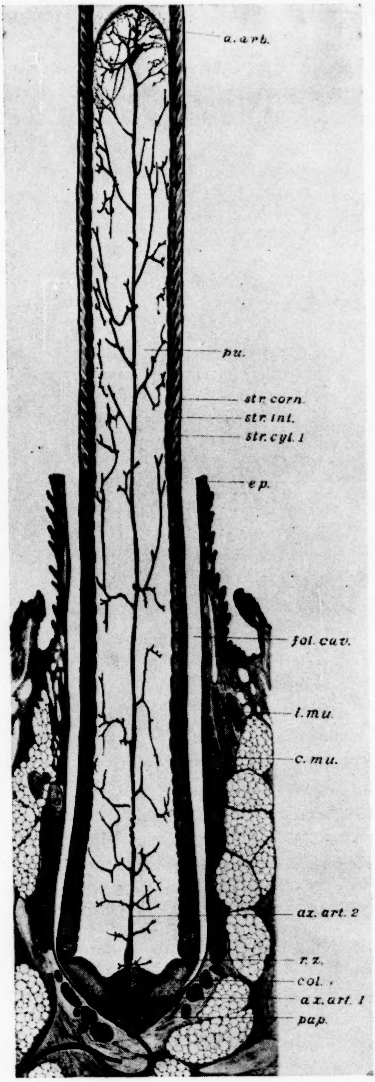

FIG. 2.—Longitudinal section of the feather cylinder and follicle on the twenty-first day of regeneration, White Leghorn cock. Semi-diagrammatic. It shows the arterial circulation in the pulp in addition to the features mentioned in the text (from Lillie, 1940). ×18.

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[3] We adopt this excellent term from 'Espinasse (1940); it includes the developing and growing epidermal part of the regenerating feather held together by the sheath in cylindrical form. In our sense it refers more specifically to the part containing the pulp. It is not a complete substitute for the common term "feather germ," which is a generalized and indefinite term similar to "embryo."

the feather has been plucked.[4] The constriction, of course, represents the position of the umbilical lip of the collar (cf. Fig. 3). The torn apical surface devoid of ectoderm is through the plane of breakage due to plucking about in the position of the broken line in Figure 3. A thin epidermal layer covers all the remainder of the papilla, beginning abruptly at the sides of the torn apex.

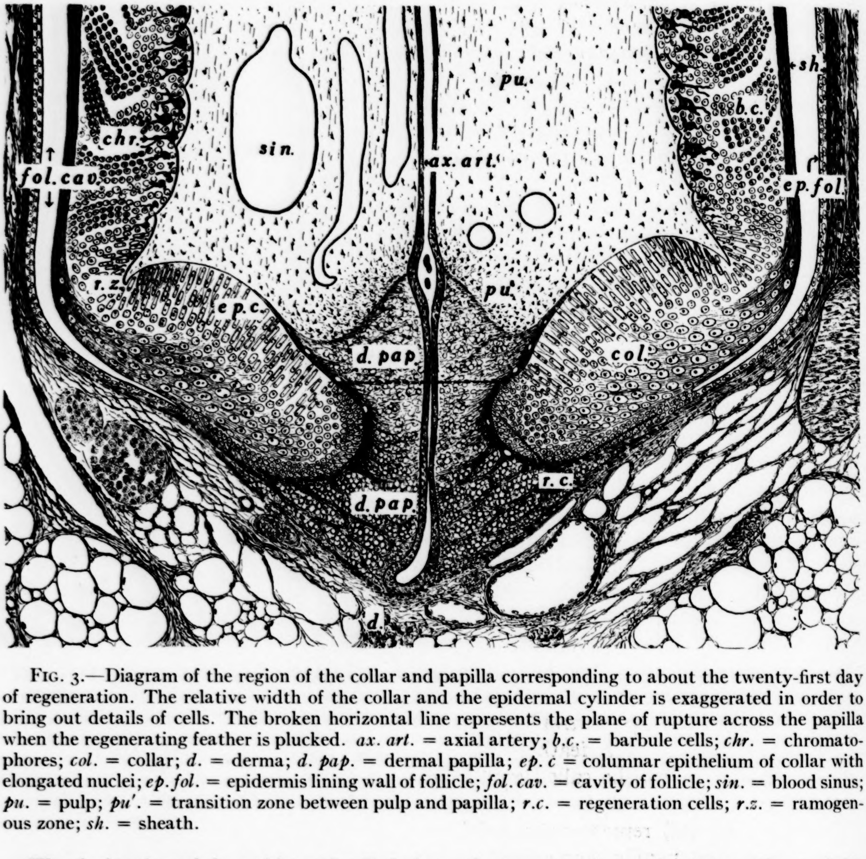

FIG. 3.—Diagram of the region of the collar and papilla corresponding to about the twenty-first day of regeneration. The relative width of the collar and the epidermal cylinder is exaggerated in order to bring out details of cells. The broken horizontal line represents the plane of rupture across the papilla when the regenerating feather is plucked. *ax. art.* = axial artery; *b.c.* = barbule cells; *chr.* = chromatophores; *col.* = collar; *d.* = derma; *d. pap.* = dermal papilla; *ep. c* = columnar epithelium of collar with elongated nuclei; *ep. fol.* = epidermis lining wall of follicle; *fol. cav.* = cavity of follicle; *sin.* = blood sinus; *pu.* = pulp; *pu'.* = transition zone between pulp and papilla; *r.c.* = regeneration cells; *r.z.* = ramogenous zone; *sh.* = sheath.

The derivation of the epidermal cells is from the regeneration cells of the collar and the wall of the follicle (Fig. 3, *r.c.*). They extend on both sides of the umbilicus as far as the boundaries of the papilla, thus, on the side of the pulp to the junction of the papilla with the pulp and on the opposite side likewise to the margin of the papilla, where they gradually thin out into the epidermal lining of the follicle. On the pulp side the regeneration cells are sharply bounded from the specialized cylindrical cells of the collar by a notch. When the feather is plucked, the regeneration cells are torn from the collar and remain attached to the mesodermal papilla, so intimate is their union, by means of a thick, structureless, basement membrane. Correspondingly, sections of the plucked feather itself through the collar show complete absence of regeneration cells over and on both sides of the umbilical lip.

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[4] Fifteen active and ten resting papillae have been studied in serial sections in order to satisfy ourselves concerning their uniformity.

The regeneration cells, which form the ectoderm of the papilla, are totipotent as far as the feather is concerned; by their growth, multiplication, and differentiation they produce a new feather complete in all its parts, under control of certain morphogenetic functions of the dermal papilla.

After regeneration is completed, the papilla lies beneath the inferior umbilicus of the feather in a resting state. The mesoderm of the resting papilla (Pl. III, Fig. 12) consists of a more cellular (basophilic) apical half and a more fibrous (acidophilic) basal half. Between the apical and the basal halves there is an ill-defined peripheral constriction. The surface of the thick mesodermal core is covered by a thin, single-layered epidermis which may, however, be deficient over the apex.

IV. EXPERIMENTS ON THE PAPILLA

1. TECHNIQUE

The following account relates primarily to feathers from the anterior portion of the breast which were used in a great majority of the experiments. Operations on feather papillae from other tracts differ only in detail. The anterior breast is an advantageous region for the operations because of its ready accessibility and because the follicles are oriented obliquely in the skin and there is relatively little fat surrounding them.

Most of the operations have been made on the active papillae of regenerating feathers from 15 to 30 days of age. Such an active papilla is larger than the resting papilla of the finished feather, and the wall of the follicle is thinner and more flaccid—both conditions that facilitate operation. However, a smaller number of operations made on resting papillae yielded results similar in principle, so that we are convinced that the resting papilla as a morphogenetic system differs in no essential way from the active papilla.

The bird to be operated on is fastened to an operating board by loose knots around the base of the wings and on both feet. It is anesthetized before operation by intraperitoneal injection of Nembutal (pentobarbital sodium, Abbott) with a dosage of about 0.5 cc. for each kilogram body weight, which puts the bird to sleep for 2 or 3 hours.

The region around the feathers selected for operation is then plucked, or the feathers surrounding the selected ones are clipped, and the bird is covered with clean, dry towels except for the operation site which is washed with 70 per cent alcohol. The feathers selected for the operations are plucked immediately before operating.

Instruments used in the operation are first washed in hot water, then left on a clean towel, and immediately before use are sterilized in 70 per cent alcohol. For the purpose of washing away blood and moistening tissues during operations, sterile Ringer's solution is used.

Operations are made under a binocular microscope arranged so that it can be swung over the site of operation. When the selected follicle is in focus, a longitudinal incision is made along its upper surface to a point near the bottom of the follicle, and the flaps are reflected. This should be done with a single clean cut.

The actual cuts on the papillae are made with the finest iridectomy scissors. With two pairs of very fine steel forceps the papilla is brought into view by grasping the wall of the follicle near the bottom and gently lifting the papilla. The iridectomy scissors are then taken up, and the operation on the papilla is made by a single cut, or as few cuts as are required to remove the parts indicated in the diagrams of operations (Fig. 4). The birds stand the operations well. Deaths of experimental birds referred to in the text were due to extraneous causes.

Operations of Type A, involving only the apical half of the papilla, are quite readily performed, because the apical half of the papilla protrudes freely into the follicular cavity and there is a distinct difference in the degree of opacity of the apical and the basal halves of the papilla. In operations of Type B, involving the entire length of the papilla, the cuts must carry through the bottom of the follicle.

It is easy to remove the entire papilla, or definite parts of it, for transplantation or recombination experiments. In such experiments part of the follicular wall is left as a means of ready handling. The papilla may be left in Ringer's solution while the transplantation site is being prepared.

We have not attempted to use marks on the skin by tattooing or otherwise for identification of papillae. The practice has been to map the positions of the operated follicles with reference to established landmarks on the bird's breast, such as clipped feathers immediately surrounding the operated ones. The fact that all operated papillae are relatively slow in beginning regeneration may be used to differentiate them from others of the same age. Moreover, the follicles of operated papillae may be distinguished by the scar along the line of healing, so that proper identification offers no difficulty.

2. INTRODUCTION TO EXPERIMENTS

All operations on the papilla are oriented as exactly as possible. Inasmuch as the papilla exhibits no structural differences except along the primary axis, the dorsoventral orientation is determined *in situ* while the operations are being performed by the relations of the papilla in the follicle to the dorsal and ventral surfaces of the feather. We can recognize *in situ* apical and basal ends, dorsal and ventral surfaces, and right and left sides of the papilla; operations can be made accordingly in transverse, sagittal, and frontal planes at selected levels. But the absence of landmarks creates a difficulty in precise localization of cuts.

Without exception, feathers derived from operated papillae are slow in emerging from the mouth of the follicle in comparison with those derived from normal papillae. The repair of the follicle, which always takes place very rapidly after the operation, does not seem to be a considerable factor in the delay. Factors inherent in the operated papilla itself seem to us more important. Among these are the effective restoration of the blood circulation, repair of the ectoderm on the wounded surface, and, presumably, certain obscure adjustments of the mesoderm of the papilla. The time of emergence of feathers derived from normal papillae is from 7 to 9 days in the breast tract. This time is increased in general to 13 to 15 days in the case of operated papillae, but in the case of removal of the apical half of the papilla (Operation *T*, Fig. 4) the time of emergence is still longer, at least 18 days.[5]

After the feather has emerged from the follicle its rate of axial growth may be approximately normal, and the length attained may be equal to that of control feathers. This happens only in case a normal rhachis develops, as, for instance, in feathers derived from dorsal halves of the papilla; but in other cases the rate of axial growth and the length attained are diminished, sometimes to an extreme extent.

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[5] Over half of the total number of operations, which exceed five hundred in number, have yielded feathers of some sort and are in that sense successful. The percentage is, of course, lower in some operations than in others.

The types of experiments are similar to those in experimental embryology: (1) "transplantation experiments," in which a papilla or a part is transplanted to a new environment, e.g., to another follicle, or subdermally outside of a feather tract; (2) "defect experiments," in which a determinate part is removed and discarded and the remainder allowed to develop; (3) "isolation experiments," in which the papilla is merely divided in a determinate way and the parts left to develop; and (4) "recombination experiments," in which parts of two papillae are brought together in the same follicle.

3. TRANSPLANTATION EXPERIMENTS: ORIENTATION AND TRACT SPECIFICITY OF PAPILLAE

Transplants of isolated papillae have been made for the most part to sites on the same individual (autoplastic); the same technique may be used between different individuals of the same breed or between individuals of different breeds, but these do not succeed so readily; they also introduce other problems that are not considered in this paper.

Autotransplants have been made within the same follicle, to follicles of other tracts from which their own papillae were removed, and to subdermal locations between feather tracts. Within the same follicle the "transplantation" is merely a rotation of the papilla around its own axis, after it has been dissected completely free, through 90° or 180°. The operation is for the purpose of determining from the orientation of the resulting feather whether or not the normal dorsoventral orientation of the feather is due to an inherent dorsoventral organization of the papilla.

In a particular experiment on a Brown Leghorn hen, after plucking the feathers, six follicles were opened along their length to near the bottom; in each the papilla was then dissected entirely free from all attachments, rotated either 180° or 90° along its axis, and then replaced. Two failed to develop (Nos. 2 and 3). Of those that developed, the ventral surface was up and the dorsal surface next to the skin in two (Nos. 4 and 6) that were rotated 180° on the long axis; in the two others, in which the papillae were rotated 90°, the feathers also were rotated about 90°.

In another experiment, on a Brown Leghorn male, that involved also the question of tract specificity of isolated papillae, seven papillae of regenerating saddle feathers were grafted into breast follicles from which the papillae had been removed, three in normal orientation and four rotated 180° axially. One of the former formed a saddle feather with normal orientation, while two failed to develop. Two of the four with reversed orientation developed saddle feathers ventral side up, while the other two failed to develop. Similarly, breast papillae transplanted to saddle follicles developed into breast feathers (three out of five cases).

Breast papillae dissected out of the follicle and free from other tissue have been transplanted subdermally in the region of naked skin between breast and dorsal feather tracts that is protected against external injury by the wing in its natural position. Subdermal blood vessels can be seen through the thin skin of this region, and a location at the angle of a branch is selected as favorable for vascularization of the implant.

Regeneration is always unusually slow in the case of such implants. This is presumed to be due to the mechanical difficulties of piercing through the skin, which happened only once spontaneously; usually the graft becomes encapsulated in connective tissue, but evidences of growth are obvious by subdermal swellings. In three cases the struggling germs were liberated by cutting through the skin, and they developed into external feathers.

From eighteen such implants of breast papillae four have developed breast feathers, which have grown through the skin and formed typical follicles. In one case the bird died before a second-generation feather could be formed; another bird died after the second-generation feather was well developed. The third bird had two successful grafts, in their third generation at the time of writing. The transplanted papillae have therefore become established. The feathers are disoriented dorsoventrally and with reference to the axis of the body, owing to the fact that fixed orientation cannot be given to the transplanted papillae.

The transplantation experiments demonstrate that the axial and the dorsoventral orientation of the feather is determined by intrinsic functions of the papilla and also that the papilla carries with it the specificities of the tract of origin.

Experiments on transplantation of skin from one feather tract to another have shown that the transplants develop feathers of the type of the tract of origin (Danforth, 1929, 1937). The present experiments show that the tract specificity of the feather resides in the individual papillae, not in the transplant as a whole. Transplantation of isolated papillae is applicable to more exact problems, as, for instance, the production of chimaera feathers (p. 118) or interbreed reactions of melanophores where the papilla is of foreign origin.

4. THE DEFECT EXPERIMENTS

A. INTRODUCTION

Defect experiments are the fundamental tests for the morphogenetic properties of the papilla. Several principal kinds have been carried out, as shown in the diagram of operations (Fig. 4). They will be designated as follows:

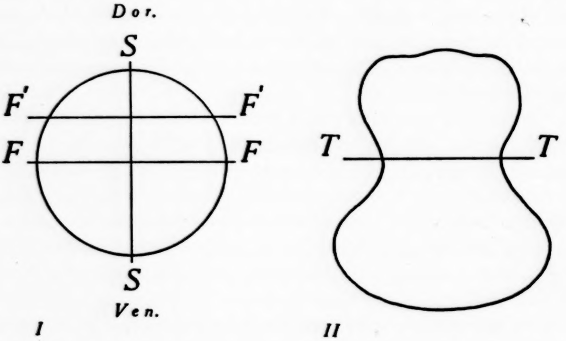

FIG. 4.—Diagram of operations. I. Cross-section of the papilla to show planes of operations F and F′, equal and unequal frontal operations, and S, sagittal. II. Side view of the papilla to show the position of the transverse operation T.

T (*transverse*).—The papilla is bisected transversely, and the apical portion discarded. The experiment tests the developmental capacity of the basal portion of the papilla. Only one level of amputation has been tested, approximately halfway between apical and basal surfaces of the papilla.

F (*frontal*).—The plane of operation is frontal. The F experiments through the center of the papilla are of two kinds, A and B; in the A type of experiment the operations are limited to the apical half of the papilla (F.A.); in the B type the operations involve the entire papilla (F.B.) and thus also a certain minimal amount of the bottom of the follicle. Each of these again presents two alternatives according as whether the dorsal (F.A.D. or F.B.D.) or the ventral part (F.A.V. or F.B.V.) is discarded. The *F′* plane was used only to determine the developmental capacity of the dorsal quarter of the papilla (F′.B.V.).

S (*sagittal*).—The plane of operation is sagittal through the center of the papilla. Here also A (S.A.) and B (S.B.) types of operation are distinguished. Either right or left parts may be discarded.

B. CLASSIFICATION AND DESCRIPTION OF ABNORMALITIES

From the defect experiments alone we have preserved and filed about one hundred and fifty feathers belonging to the first generation after operation. These present an astonishing array of abnormal feather forms, no two of which are precisely alike. All, however, are composed, so to speak, of certain "elementary abnormalities" of the rhachis and barbs. The same elements of abnormal structure also occur in isolation and recombination experiments. They may be classified as follows.

I. Elementary abnormalities of the rhachis
  1. *Absent.*—Absence of the rhachis may be of any degree from apex to base; it never occurs in an intermediate position. A "tuft feather" results (Pl. IV, Figs. 18 and 19; Pl. VIII, Fig. 28).
  2. *Halved.*—One lateral half of the rhachis is missing,[6] a condition that likewise occurs in variable extents from apex to base and not in intermediate positions. A half-vane type of feather results (Pl. V, Figs. 20 and 21).

II. Principal elementary abnormalities of the barbs
  1. *Branched.*—One or more dichotomous divisions of a single barb base (Pl. III, Figs. 13 and 14, *et passim*).
  2. *United.*—Two or more barb bases united apically (Pl. VII, Fig. 26).
  3. *Bundled.*[7]—A complex of barbs with apical branching from an intermediate union of separate barb bases (Pl. VII, Figs. 25 and 26; Pl. VIII, Fig. 29).
  4. *Reversed.*—A barb attached by its apex to a half-rhachis on the side opposite to the vane (Pl. V, Figs. 20 and 21).

Barbs also exhibit many other abnormalities not included in this list. For instance, the serial order on the rhachis of two barbs may be reversed at the margin of the feather so that the barbs cross at some point but are otherwise interlocked like normal barbs (see Pl. VII, Fig. 25, left side). This phenomenon is common in natural (Section IV, 9), as it is also in experimental, feathers. Occasionally the crossing may involve three or even four barbs. Crossed barbs are frequently, perhaps always, bent. In the experimental feathers barbs may be stubby and much shorter than their neighbors or attenuated and much longer; they may be of much more, or much less, than normal diameter. Examples may be seen in the photographs. Abnormalities of barbules also occur.

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[6] For this interpretation strictly anatomical criteria pertaining to the rhachis itself are uncertain. The designation "halved" is derived from the organization of barbs on one side only into a typical half-vane and the lack of any such organization of barbs on the other. It is essentially a functional interpretation.

[7] The term "bundled" is used in this paper to denote only cases in which there is actual fusion of the stems of the barbs concerned to such an extent that the individuality of the component elements is apparently lost in the common section. Theoretically, however, there may be all degrees of approach to this condition from mere contact of barb stems through stages of partial fusion. Such cases are certainly extremely rare in the experiments that form the subject of this paper; but we have found numerous examples along fault bars in certain unpublished experiments of a different kind. The number of apexes concerned in such cases always agrees with the number of bases and the individuality of the barbs is not lost (cf. p. 125).

We shall, however, lay emphasis on the four principal elementary abnormalities of the barbs listed above. These are correlated with abnormalities of the rhachis. When the rhachis is absent, the barbs develop in the form of a tuft in which it is difficult to recognize any bilateral distribution (Pl. VIII, Fig. 28; Pl. IV, Figs. 18 and 19). Such feathers develop in the wall of a cylinder containing pulp, as in normal feathers, and complete their growth with resorption of pulp over a longer period of time than normal. The number of barbs produced is, however, much less than in a corresponding normal feather; counting all apexes, the total number is about one hundred and twenty-five in the specimen shown in Figure 18 (Pl. IV), as compared with an average of five hundred and forty in ten normal breast feathers of the Brown Leghorn capon. The barbs may attain excessive lengths, and, when growth is completed, no barb is of the pure vane type, but all have fluffy bases or are completely fluffy. From this it is clear that any barb will change from vane-type to fluff-type if its period of development is sufficiently prolonged and, by corollary, that union with the rhachis is required to terminate the growth of any barb, which otherwise is unlimited within the period of development of the feather. We interpret the tuft feather as one in which growth of the barbs has been indefinitely continued in a strictly axial direction.

In Figure 28 (Pl. VIII) the rhachis is present in the cylindrical part of the feather; in Figures 18 and 19 (Pl. IV) the rhachis is certainly much diminished, perhaps entirely absent in Figure 19, although the calamus is formed.

If one lateral half of the rhachis is missing, as in Figures 20 and 21 (Pl. V), the barbs are abnormal on the missing side and normal on the opposite side, according to the length of the half-rhachis, thus producing the "half-vane" type of feather, of which we have numerous examples. The barbs of the defective side are similar to those of feathers lacking the rhachis entirely, except that those next to the half-rhachis are usually of the extraordinary type of "reversed" barbs, i.e., attached by their apexes to the half-rhachis. Reversed barbs may have free bases, or the bases may be in the feather cylinder or attached to the same half-rhachis ("looped," Pl. III, Fig. 17).

Operations involving the apical half of the papilla alone usually produce feathers with "apical defects," limited to between 1 and 2 cm., the remainder of the feather being normal. Examples are shown in Plate III (Figs. 13–17), and in Plate VII (Fig. 26). All classes of barb abnormalities may be found in the defective region; they are more pronounced on one side than on the other, which may, indeed, bear only normal barbs as in Figures 15 and 16. The correlated abnormality of the rhachis resembles the type of halving rather than of absence, even to the extent of occasional presence of reversed barbs (Fig. 17).

We have thus distinguished three principal types of abnormal feathers—tuft feathers, half-vane feathers, and those with apical defects only. There are also occasional specimens not readily classifiable under these types, including several veritable "museum pieces." A segment of one of the latter is shown in Plate VII (Fig. 27). Such cases also are composed of the same elements of abnormal structure as the principal types.

C. RELATION BETWEEN TYPES OF OPERATION AND RESULTS

In this section we shall correlate the types of abnormal feathers produced in the defect experiments with the main types of operation (Fig. 4).

1. *Experiment T.*—The apical half of the papilla is amputated. The location of the cut is approximately in the center of the constriction corresponding to the lip of the umbilicus (Fig. 4). The amount of the papilla removed is subject to slight fluctuations.

Nine feathers of the first generation resulted from this experiment on active papillae. Seven of these have apical defects only, as illustrated in Plate III (Fig. 15) for one case; the defective region in this case is 10 mm. long, or about 12 per cent of the total length of the feather; in the other six cases of apical defects derived from this experiment the elementary abnormalities differ, and the length of the defective region varies from 5 to 11 mm. Thus it becomes evident that the basal half of the papilla is capable of producing about 90 per cent of the length of the feather in normal fashion.

Of the two exceptional feathers derived from this experiment, one was dwarfed but essentially normal in structure; the other also was dwarfed (only 5 cm. long) and highly abnormal on one side (Pl. VII, Fig. 27).

The same experiment on resting papillae yielded seven feathers, of which five showed apical defects of varying extent, and two were approximately normal.

2. *Experiments F and F′.*—There are five varieties of defect operations in the frontal plane: F.A.V., F.A.D., F.B.V., F′.B.V., and F.B.D.

a) *F.A.V.*—The ventral half of the apical half of the papilla is removed. We have five first-generation feathers resulting from this operation, of which three are normal feathers except that the apexes are narrower than in the controls and slightly bent laterally; one has very minor apical defects; the last has greater apical defects.

If these results be compared with the T experiments, agreement will be noted in limitation of defects to the apex of the feather, but normal apexes also occur in the F.A.V. experiment in three out of five cases. This suggests that the ventral part of the papilla is not needed for the normal development of the rhachis.

b) *F.A.D.*—The dorsal half of the apical half of the papilla is removed. We have twenty-six first-generation feathers resulting from this experiment. Three of these are nearly normal throughout, sixteen have apical defects only, and the remaining seven are abnormal throughout like F.B.D. experiments. The group of sixteen resemble the T experiments both in having defects limited to the apex and also in the defective characters of rhachis and barbs, from which we may conclude provisionally that the dorsal surface of the apical half of the papilla is necessary for normal development of the rhachis. The remaining seven with defects along the entire length of the feather are to be explained by removal of a greater portion of the dorsal surface of the papilla than intended (cf. F.B.D.).

c) *F.B.V.*—The entire ventral half of the papilla is removed, and the dorsal half develops alone. We have four first-generation feathers derived from this experiment on active papillae, of which three are approximately normal; in the fourth the vane portion is abnormal, carrying two or three reversed barbs, but the region of the fluff is normal. The normals were strong, growing feathers with rate of growth approximately equal to the control. The aberrant feather can be explained by injury to the dorsal surface in the operation. From the same experiment on resting papillae five feathers with normal shafts were derived, and one dwarf vane. We may thus conclude that the dorsal half of the papilla is capable of producing normal feathers.

d) *F′.B.V.*—Three-quarters of the ventral portion of the papilla is removed, and the dorsal quarter develops alone (Fig. 4, F′).

This experiment was made for the purpose of locating more precisely the primordium of the rhachis in the papilla. We have ten first-generation feathers resulting from this experiment on active papillae. Six of these are nearly normal, one shows typical apical defects, and three are highly abnormal. The dorsal quarter, like the dorsal half, produces strong growing feathers, with the exception of the highly abnormal minority. The ventral three-quarters of the papilla is not necessary for the development of a normal rhachis.

e) *F.B.D.*—The dorsal half of the papilla is removed, and the ventral half develops alone.

The development of the ventral half of the papilla offers the greatest contrast to that of the dorsal half. We have thirteen first-generation feathers derived from this experiment on active papillae; four of these are tuft feathers lacking the rhachis entirely, six are of the half-vane type (Pl. V, Fig. 20), two have a rather long, defective apical region resembling the F.A.D. type, and the last was a much delayed dwarf feather of no regular type. Thus the characteristic result of this experiment is absence of the rhachis or its development on one side only, always reduced in length.

From the same experiment on resting papillae six tuft feathers of varying degrees (cf. Pl. IV, Fig. 19, and Pl. VIII, Fig. 28), three half-vane feathers and one feather with apical defects were derived. There was thus a larger proportion of tuft compared with half-vane feathers than in this experiment on active papillae.

3. *Experiment S.*—There are three types of defect operations in the sagittal plane; in one (S.A.) the attempt was made to remove only a lateral half of the apical half of the papilla; in the second (S.B.) a lateral half of the entire papilla was removed; in the last, sagittal thirds were tested.

a) *S.A.*—We have five feathers resulting from this experiment; three of them are normal or with very minor apical defects; the two others, for some unknown reason, are quite highly abnormal on one side.

b) *S.B.*—We have seven feathers resulting from this experiment. In them all, the feathers tend to be normal except for a variable part of the apex involving one-ninth to one-quarter of the total length of the feather. We conclude that a lateral half of the dorsal surface of the papilla may produce a normal rhachis.

c) Three experiments were made to test the width of the rhachis field of the papilla: (*a*) In the first experiment a sagittal cut removed about two-thirds of the papilla on the right side, leaving one-third on the left. (*b*) In the second, a median strip of the papilla was removed, leaving two lateral strips. (*c*) In the third, lateral strips were removed, leaving a median strip about one-third the width of the papilla. Six operations of each kind were made.

The details of the results of these experiments are rather confusing, but the outstanding result is that a complete rhachis may develop from either a lateral, or the central, one-third of the dorsal surface. It thus becomes quite certain that the rhachis "field" occupies all the dorsal half of the papilla.

Summary.—From the various defect operations in the three planes there emerges very clearly the conclusion that the dorsal portion of the papilla, including both ectoderm and mesoderm, is necessary for the development of a normal rhachis and feather. Even a dorsal quarter may compensate for absence of the remainder of the papilla. On the other hand, a ventral half cannot compensate for absence of the dorsal half and does not develop a normal rhachis. The T experiments (Fig. 13, Pl. III) and the other operations involving only the apical half of the papilla (A type) clearly demonstrate an axial organization of the papilla also.

5. THE ISOLATION EXPERIMENTS

In these experiments the attempt was made to isolate parts of the papilla from one another by sagittal and frontal cuts along its entire length in the hope of producing two or more feathers in one follicle from a single papilla. Sagittal bisection yielded feathers from thirty-eight follicles, frontal bisection yielded feathers from twenty-five follicles, and division of the papilla into quadrants by both sagittal and frontal cuts yielded feathers from sixteen follicles.

In such experiments, as the parts remain in contact by wounded surfaces, there will be a tendency for them to heal together before actual regeneration begins. This was a very common condition. In a large number of cases only one of the isolates survived and developed; this was very clear in the case of frontal bisection because the dorsal and ventral isolates yield different results, as shown by the defect experiments; but in the case of sagittal isolates the distinction between complete healing-together and development of one half only is not easy to make.

In the case of frontal bisection the dorsal isolate usually suppresses the ventral one on account of the stronger growth of the former. Results do not differ from those of defect experiments. Similarly, the double bisection does not yield any results peculiar to itself. We therefore confine our attention to the results of sagittal bisection.

In this case, judging from the defect experiments, there is the possibility of producing two similar feathers in one follicle. There is no evidence of this in twenty-seven cases; but in the remaining eleven cases partial or complete twinning occurred. Two of these were complete twins from base to apex; in the remaining nine cases the feather pairs were more or less fused together from the base in varying degrees toward the apex, never in the reverse direction.

The specimen on the left in Plate VI (Fig. 24) is a photograph of one of the cases of complete twinning made in an early stage of development on the living bird. It will be observed that two feather cylinders, each with a separate sheath, emerge from a single follicle. These developed into separate feathers of unusual length (11 and 9.8 cm., respectively). The second case of complete twins show mirror imaging of brown areas in an otherwise black vane. In the other cases there was a single sheath for both components as in the twin pair on the right in Plate VI (Fig. 24). In such cases the twinning may be complete to the calamus (one specimen), to the fluff (one case), or in part of the vane only.

Figure 23 (Pl. VI) shows a case of twinning to the fluff region, which was plucked early for fear of losing it. The two rhachises approach each other and are apparently about to fuse in the common sheath. The vanes are not entirely normal in either partner but more nearly so in the right one. It will also be noticed that the external half-vanes of both partners are more nearly normal than the apposed vanes, owing presumably to interference of the latter with one another during development.

A very beautiful case of apical twinning is shown in Figure 22 (Pl. VI). The successive generations of this feather will be discussed later (p. 121).

6. RECOMBINATION EXPERIMENTS: CHIMAERAE

Feather papillae have the capacity for independent self-differentiation and can exhibit this capacity outside of their natural feather tracts. We have also seen that lateral halves of one sagittally divided papilla tend to reunite; the expectation of a union of lateral halves from different tracts, if the cut surfaces are apposed, is therefore not unreasonable. It should be possible, if technical difficulties can be overcome, to combine opposite lateral halves of papillae from different tracts on the same side of the body into a single papilla that will produce a feather with one half of the vane derived from one tract and possessing its characteristics, and the other half derived from the other tract and possessing its characteristics—in short, to produce a lateral feather chimaera.

Breast feathers and saddle feathers of the Brown Leghorn capon offer contrasting characters that suit them admirably for such an experiment (Pl. I, Fig. 6). The barbs of the breast feather bear barbules to their extreme tips, those of the saddle feather bear them only near their bases, and the remainder of the barb is devoid of barbules, giving the feather a "lacy" effect; the extent of the portion of the barbs bearing barbules in the saddle feather increases very gradually from a minimum apically until, near the region of the fluff, about a half of the length of the barb bears barbules. The color of the breast feather in the capon is blackish, that of the saddle feather golden brown. The fluffy region of the breast is relatively longer than in the saddle feather. The breast feather is broader and more rounded at the tip; the saddle feather narrower and acuminate.

The experiments were made by sagittal bisection of a breast papilla and removing one half as in the defect experiments, then transferring the opposite lateral half of a saddle papilla from the same side of the body and pressing its cut surface against the cut surface of the breast papilla in homologous orientation within the bottom of the opened breast follicle. The follicle repairs itself as usual.

Two experiments have been made. In the first experiment five saddle components were grafted to contralateral components of the breast tract. In the second, four reciprocal transplantations between breast and saddle tracts were made. Thus there were thirteen combinations in all. From these three typical lateral chimaerae were derived. Three breast follicles produced breast feathers, two saddle follicles produced saddle feathers, one saddle follicle produced both a breast and a saddle feather; three of the remaining four follicles produced feathers showing evidences of double composition, and the last feather developed only fluff.

One of the lateral chimaerae is shown in Plate I (Fig. 7). Its form is more like that of a saddle than that of a breast feather. The barbs on the left side are provided with barbules to their tips; and the pigmentation, though not so black as that of a normal breast feather, is darker and different in tone from that of the opposite side, which has the typical golden-brown color of the saddle. The vane on the right side is lacy, with axial distribution of barbules typical of a normal saddle feather. The fluff region is longer on the left than on the right side, corresponding to the normal proportions in breast and saddle feathers, respectively. At the apex there is not the least transition between breast-barb and saddle-barb, but typical ones are side by side. The second lateral chimaera differed mainly in the distribution of barbules, which was wider than usual on the saddle side; in the third the breast component is quite black.

Each component of the papilla in these cases has preserved its own tract specificity and field of action, but the whole constitutes a unity due to certain interchanges of effect between them. It would hardly be suspected, for instance, by observation of the chimaera alone that it was composed of half of a relatively short and broad feather and half of a relatively long and narrow feather (Pl. I, Fig. 6). There has been mutual accommodation of the components to produce a symmetrical result.

Danforth (1929 and 1937) described mosaic feathers near the margins of skin transplants between homologous feather tracts of different breeds of fowl. The effect was confined to color pattern and reappeared in successive generations. He rejects as inadequate the idea that "appropriate parts of two cut follicular anlagen were fortuitously brought into direct apposition" (Danforth, 1929) and suggests the possibility that nearby foreign tissue may in some way exercise a determining function. In his later paper (1937) this idea is further refined into the suggestion that migration of breed-different chromatophores may have been involved, which gains great probability from the later beautiful study of Willier and Rawles (1940) on the determination of feather pattern in the host by migration of the precursor cells of chromatophores from embryonic grafts. The technique employed in our experiments could well be adapted to further study of this problem.

7. SOME SPECIAL EXPERIMENTS

A. In sixteen cases in the breast tract the apical half of the papilla was reimplanted in its own follicle after complete removal of the basal half. Of eight cases in which a White Leghorn female was used, four did not regenerate; after 40 days two produced only a dwarfed group of vanelike barbs, and two produced portions of the vane 2.2 cm. and 2.7 cm. in length, respectively, which were not entirely normal. Of eight cases in which a Brown Leghorn male was used, six failed to regenerate; after 70 days one produced an approximately normal feather vane 4.5 cm. long, the other a half-vane feather 4 cm. long.

In five cases the apical halves of papillae of the breast tract were transplanted to follicles of fluff feathers dorsal to the breast tract from which their own papillae had been removed. Three failed to regenerate; after 52 days one produced a breast-type vane 3.2 cm. long that was approximately normal; the remaining one produced a short tuft of vanelike barbs without rhachis.

From these experiments it is obvious that the apical half of the papilla experiences considerable difficulty in establishing itself in viable condition, but, when it does so, its developmental capacity may be considerably more than its apparent prospective value judged from the defect experiments.

B. Dorsal halves of two papillae from the breast tract were applied together by their cut surfaces in the follicle belonging to one of them; thus in one partner the dorsoventral orientation is preserved; in the other, reversed. Out of five combinations one produced two approximately normal feathers 5.5 cm. long with ventral surfaces apposed, thus according to expectation. Three produced single normal feathers, and the fifth a dwarfed single feather with the rhachis forked about the middle of its length.

C. Ventral halves of two papillae from the breast tract were applied together by their cut surfaces in the follicle belonging to one of them. Out of five combinations, one produced two feathers of the half-vane type each 6.8 cm. long; another produced two feathers, of which one was of the half-vane type 8 cm. long and the other a typical tuft feather without rhachis 5 cm. long; and two produced single tuft feathers.

D. Kuhn (1932) produced a spiral twist of 180° in wing primaries of the pigeon by the following experiment: After plucking normal primaries, the long follicles dissected free nearly to the base were constricted by a thread tied tightly near the mouth of each, twisted several times, and bound by the free ends of the thread to neighboring primaries so as to preserve the twist. Although the follicles degenerated and were replaced by new ones, the feathers that resulted showed a half-spiral of rhachis and vanes with normally oriented base and the tip 180° reversed. Not being acquainted with the papilla, Kuhn attributed the result to distortion of the "matrix" from which the feather is derived. The condition persisted for three generations and gradually disappeared thereafter.

We have not succeeded in similar experiments on the Brown Leghorn but have produced similar spiral feathers in other experiments.

a) In a Brown Leghorn male the dorsal half was removed from three papillae of wing primaries (Experiment F.B.V.). In one case a half-vane feather resulted, repeated in the second generation now growing. This was an expected result. In the second feather, which attained a length of 16 cm. before plucking, the apex was twisted 180° at a mean distance of about 2 cm. from the tip. The second generation was similar, but the twist was somewhat reduced in the third generation now growing. The third feather was slightly twisted.

b) In a Brown Leghorn female three papillae of wing primaries were bisected sagittally. In one of these twin feathers developed. The second started as an apical twin, but one partner broke off; and the other, which attained a length of 15 cm., was twisted 180° at a mean distance of 4.5 cm. from the apex; the twisting was lost in the second generation. The third was a single feather rotated 180° at a mean distance of 5 cm. from the broken apex; the second generation, which attained a length of 19.5 cm. was likewise rotated 180°, but at a mean distance of only 3.5 cm. from the apex.

There are thus four cases of twisting out of six operations on wing primaries of the Brown Leghorn fowl in which the division of the papilla was longitudinal. Such cases have not been found in the much more numerous operations of similar nature on papillae of the breast tract. Although the papillae of the primaries are much larger than those of the breast tract, they are more slender in their proportions; they are also more difficult to get at. It may be that these conditions combine to give an unintentional twist to the papilla in the act of operating, but this is, of course, uncertain.

It is noteworthy that the operations were all on the left wing and that the direction of the spiral is the same in the four cases, which we designate as counterclockwise.

c) We attempted to produce spiraling in the breast tract by almost, but not completely, amputating the apical half of the papilla and rotating it 180° on the base and grafting it back. One out of eight feathers derived from this experiment showed a 180° spiral centered at the junction of the vane and the fluff, in addition to numerous elementary abnormalities of the barbs. The others exhibited the defects but not the spiral. The single case is, of course, not accidental. The twist disappeared in the second generation; the bird died after this.

Spiraling of the feather must be traced back to spiraling of the papilla if the results are to be interpreted in agreement with the other experiments. This interpretation has important theoretical consequences. It is noteworthy that the spiraling persists from only one to three generations and offers a contrast in this respect to other abnormalities (see Sec. 8 of this paper).

8. LATER GENERATIONS OF FEATHERS FROM OPERATED PAPILLAE

When a feather from an operated papilla is plucked for record, the papilla then proceeds to regenerate a new feather. These are likewise plucked and filed when mature, and so on for successive generations. The collection is, however, far from complete; when some of the birds ran at large in the summer the feathers were not plucked, and whole series were occasionally lost owing to death of a bird which might bear a considerable number of cases.

Among the defect experiments we have twenty-three cases in which both first and second generations have been preserved; in seven of these we have also the fourth generation. There are also seven additional cases in which first and third generations have been preserved. In these cases the general type of defect is repeated from the first to the fourth generations; defects are almost always more pronounced on one side, which remains the same from generation to generation. The details, but not the type, of the defects vary from generation to generation; but there seems to be no tendency toward a diminution of effect in four generations.

This result is so striking, and to some extent so unexpected, that individual cases deserve special attention. It might be expected that in the case of the T operation, which involves only amputation of the apical half of the papilla, the remainder would regenerate the missing part and that normal feathers would then be produced. However, the apex of later generations of such feathers remain defective. We have six cases in the second generation, after which the bird died, and two cases in the third generation from another bird. We have also five additional cases of F.A.D. operations in the second generation and two in the third generation in which the apical defects are repeated, and, finally, two cases of this experiment that produced limited half-vane type of defects in both the first and the second generations.

In the F.B.V. and S.B. experiments, which produce approximately normal feathers, those of the second generation are likewise approximately normal in the three cases recorded.

Seven half-vane feathers of the F.B.D. experiment were replaced by half-vane feathers both in the second and in the fourth generations (the third generation having been lost while the birds were running at large during the summer). In each case the half-vane was on the same side of the feather in successive generations. The photographs of first and second generations of one of these cases (Pl. V, Fig. 20) illustrate also the principle that the type of defect remains constant but details differ in successive generations; the half-vane is shorter in the second generation, and the free reversed barbs present in the first generation are lacking in the second generation.

The twins and chimaerae illustrate particularly well the persistence of composition of papillae established by operation. The four generations of the apical twin shown in Plate VI (Fig. 22) are strikingly alike in all major features. The rhachis is double from the fluff up, and it forks in two branches at approximately the same level in all. If the tips of the feathers appear different, this is due solely to differences in the length of the barbs in the apposed vane-halves in succeeding generations and to consequent variation in the degree of overlapping.

The chimaera illustrated in Plate I (Fig. 7) is of the second generation, which is practically identical with the first, and also with the third not yet plucked.

In general, the observations on the later generations demonstrate a surprising lack of capacity for regulation on the part of the papilla, except in the case of spiraling. It is possible that still later generations may demonstrate slow regulation, and the birds will be kept under observation as long as possible to determine the question.

9. THE OCCURRENCE OF ABNORMALITIES IN NATURAL FEATHERS

The abnormalities that we have produced by experiments have not hitherto been described as occurring in natural feathers. From this it may be concluded that they are at least very rare. We have examined over thirty-five hundred breast feathers from thirty-six Brown and White Leghorns and found that an average of 6.6 per cent of the feathers exhibited branched and bundled barbs. If branching at the extreme apex is eliminated for reasons stated below, the percentage in over two thousand feathers specially examined for this point was reduced to 1.7 per cent. The other principal elementary abnormalities of barbs were practically entirely absent.

Individual birds varied to an extreme degree in the percentage of feathers exhibiting these abnormalities. When the reasons for this variation were examined, we found that, out of a total of nine hundred and ninety-five feathers from seven birds not used in experiments and not previously plucked, only twelve showed occurrence of these abnormalities which were confined to the apex in ten cases; no abnormal feathers at all were found in three of these birds. In the remaining birds the percentage of abnormalities was greater; all the feathers examined were known to have regenerated within previously plucked areas; the birds had also been handled a great deal by strapping to the operation board during repeated examinations. Of about twenty-five hundred feathers from these birds a total of 9 per cent showed the abnormalities in question, which was reduced to 3.1 per cent when branching at the extreme apex was eliminated.

The general result of these examinations is to emphasize the extraordinary precision of the mechanism of development of feathers when naturally molted. After artificial plucking, the percentage of total abnormalities is multiplied at least seven times. The preponderance of apical abnormalities would seem to indicate that artificial plucking, which always involved some partly grown feathers, tends to produce slight injuries to the apex of the papilla. Even apart from this it would seem probable that the development of the feather may be slightly deranged by continual handling of the birds, which involves the danger of mechanical injury to the papilla.

10. SUMMARY OF RESULTS

1. The rhachis consists of a central, or medullary, component and two lateral, or ramic, components composed of expanded barb-petioles united serially by their fibrous extensions. The central rhachis itself is double in origin and to some extent in structure. It is prolonged apically a minute distance by the fused bases of the first two apical barbs.

2. Experiments were performed by operations on the papilla. Active papillae of regenerating feathers were used principally, but the resting papillae of completed feathers yielded similar results.

3. Transplantation experiments of various kinds prove that the papillae preserve an innate bilateral organization in whatever orientation or location they may be placed. Papillae of breast and saddle feathers carry their tract specificity when transplanted outside their own tracts.

4. Feathers with abnormal apexes develop from the basal half of the papilla after the apical half is removed by amputation. Other operations on the apical half of the papilla also produce apical defects of the feather.

5. Approximately normal feathers may develop from the dorsal half, the dorsal quarter, or a lateral half or even a third of the papilla, after removal of the remainder of the papilla in each case.

6. Tuft feathers with no rhachis, or half-vane feathers with a half-rhachis, are the typical products of development of the ventral half of papillae from which the dorsal half has been removed.

7. Similar twins may be produced by sagittal bisection of a papilla; all grades from apical duplication to complete duplication may result, according to the amount of healing after operation.

8. A single chimaera feather possessing characters of a breast feather in one half-vane and of a saddle feather in the other half-vane may be produced by fusing contralateral halves of a breast and a saddle papilla in one follicle.

9. Some of the above findings were confirmed by certain special experiments.

10. Operated papillae continue to produce the same types of abnormal feathers during successive generations of regenerated feathers.

V. THEORETICAL

1. INTRODUCTION

The mechanism of development of the feather is normally very precise, and the results in the development very constant. The experiments on the papilla derange the mechanism to a variable extent and with variable consequences. Previous deductions concerning the mechanism have been based mostly on two kinds of data: (1) on analysis of the normal development and of feather patterns and (2) on certain experiments dealing with the effects of hormones and conditions of nutrition and growth on the development and pattern of the feather. The present experiments differ inasmuch as the materials and conditions of development are kept as normal as possible and the mechanism itself is deranged.

The explanation of the abnormalities that we have described in the defect experiments is to be found in modifications of the normal development. The ideal way would be to follow the details of development of each type of abnormality; but we have not found it possible to do this. Even if this method were followed to its practical limit, the role of theoretical interpretation would not be greatly diminished; and it is possible, as a matter of fact, without in any way exceeding the limits of established biological principles, to make satisfactory correlations of the abnormalities with normal processes of development.

2. THE ELEMENTARY ABNORMALITIES

A. THE RHACHIS

Study of the normal development shows that the central rhachis and the barbs are of entirely different origin. The experiments also are conclusive concerning the distinction between central rhachis and barbs. Nevertheless, the strict correlation of abnormalities of the barbs with abnormality of the central rhachis in the experiments demonstrates that the latter is the principal factor that controls the tangential movements and the normal behavior of the barbs in development. The interpretation of the elementary abnormalities of the barbs is, therefore, to be sought mainly in the abnormalities of the rhachis produced by the operations. In natural feathers, however, elementary abnormalities of barbs of a relatively simple kind may occur in the presence of a normal rhachis (p. 122); they appear to be associated with localized disturbances of tangential movements independent of the rhachis, judging by correlated modifications of spacing of the barbs concerned.

In our classification we have reduced abnormalities of the rhachis to two main types, viz., absence of both right and left sides and absence of one lateral half; both are graded as to extent in an apicobasal direction. On a priori grounds other types of abnormalities would be possible, such as isolated lateral (ramic) components or multiple rhachises. But it is very doubtful that these occur, though it is true that repeated branching, union, and bundling of barbs may simulate multiple conditions of the rhachis (cf., e.g., Pl. VII, Fig. 27).

Bilateral absence of the rhachis in any degree is readily diagnosed by the tufted arrangement of barbs above the level where the rhachis terminates and by the regular bilateral arrangement of barbs proximal to this point (Pl. VIII, Fig. 28, and Pl. IV, Fig. 19). Diagnosis of a half-rhachis rests on the regular arrangement of barbs to form a half-vane on one side and the reversed and tufted arrangement of barbs on the opposite side. Two independent half-rhachises do not form separately in our experience. Incompleteness of fusion of lateral halves does occur, however.

None of the various forms of branching, union, or bundling of barbs produces a structure that has more than superficial resemblance to the central rhachis, which has the unmistakable properties of attracting barbs to itself, of stimulating the formation of barb petioles, and of independent basal growth and enlargement. The experiments thus demonstrate that the central rhachis is an independent segregate with properties peculiar to itself.

B. THE BARBS

Each barb is an independently growing and differentiating unit. Cell division is confined to a very short segment at its base; above this zone keratinization sets in very rapidly and fixes conditions so that branching, union, bundling, and inversion of barbs becomes impossible. The modifications of normal development that lead to abnormalities are located in the zone of cell division. But this zone itself must be further subdivided; in its more distal section the segregation of cells for stem of the barb, for barbules, and other purposes is accomplished, and their further multiplication occurs. Proximal to this it is necessary to postulate in each barb a center of undifferentiated cells in juxtaposition with the collar from which all the various cells of the barb are derived. This we shall call the *organization center of the barb*.

The individual barbs concerned in the abnormalities are complete as to organization, which could not happen if the abnormalities arose after cell differentiation had occurred. We conclude, therefore, that the elementary abnormalities owe their origin to behavior of the organization centers of the barbs.

In normal development the bases of the barbs are set on the collar as closely together as is consistent with their mutual independence (cf. numerous photographs in the paper by Lillie and Juhn, 1938). It is one of the theses of the present paper that the normal regular spacing of the barbs is interfered with either by defect or by excess under the conditions produced by the experiments.

"Branching" will occur if two adjacent organization centers fuse and then continue to act as one; the two original barbs will be carried away from the collar by the axial growth of the whole cylinder and will be attached as branches to a single barb derived from the fused organization center. Repetition of this process involving the bases of such branched barbs and others will lead to complex branching systems (Pl. III, Fig. 14, and Pl. VIII, Fig. 29). The form of the branching will vary according as combinations of single, double, triple, or more complex centers are concerned.

"Union" will occur between barbs if the organization center of a single barb divides and the two centers then grow independently. Repeated division may then produce systems of considerable complexity (Pl. VII, Fig. 27). Union is very common as a sequence to branching, i.e., to previous fusion of organization centers, thus producing bundling. The bases of reversed barbs also are usually divided repeatedly. Union is thus common in association with other abnormalities, but it is also found separately, though rarely (cf. Pl. VII, Fig. 26).

"Bundling" will occur if fusion of two centers is followed by division of the conjoined center. The time interval between fusion and division will determine the length of the common section between the branches and the united barbs. In the case illustrated in Figure 26 (Pl. VII), three apexes of barbs are concerned and only two bases, from which it would follow that the number of organization centers entering into a fusion does not determine the number of subsequent divisions of the fusion center.[8] Fusion appears usually to produce a center of independent value. This conclusion is supported by a large number of complex cases. The repetition of bundling along an isochrone, as shown in Figure 29 (Pl. VIII) and observed in other cases, is suggestive.

The causes of three of the four major barb abnormalities are reducible to two simple biological principles of very wide occurrence, viz., the production of a single undifferentiated primordium by fusion of two, and the production of two primordia by division of a single undifferentiated primordium. What are the underlying conditions created by the operations that induce these unusual phenomena?

They are associated, as we have seen, with absence either of both halves of the rhachis or of one lateral half only and with the extent of these defects, which are graded in different cases from its apex toward its base. It is always the apex of the rhachis that is first affected; there is consequently delay in tangential movements of barbs until the rhachis forms. During the delay barbs grow vertically side by side. If the collar should retain its primitive capacity to form new barb apexes at any locus, and new ones continue to arise, irregularities both in order of origin and in spacing of barbs might be expected, owing to the altered conditions. Abnormalities may be referred to such irregularities.

Such a general explanation would remain purely formal if we were not able to deduce from the structure of the feather the order of origin of its barbs. When a vane or half-vane is present, the order of origin is the same as the order of succession on the rhachis. When the rhachis is absent and the barbs grow vertically in consequence, the relative lengths of the barbs is the clue to the order of their origin according to the principle of equal rates of axial growth around the cylinder.

We may test this out on Figure 17 (Pl. III), for example. On the defective side we have a complex of branched and bundled barbs attached by a single base to reversed barbs. The complex is much longer than the reversed barbs and, therefore, of correspondingly earlier origin. But the reversed barbs took their origin between the complex and the rhachis, that is, in a more dorsal position on the collar, which is the exact opposite of the normal order of origin. Tuft feathers and the defective side of half-vane feathers furnish additional examples.

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[8] By contrast, in natural feathers, where we have examined numerous cases of bundling (which have never involved more than four barbs), the number of the apexes concerned always agrees with the number of bases. Moreover, the arrangement of the apexes is always repeated in the bases but as a mirrored image, i.e., in inverse order. The reason for this difference is obscure.

There can be no doubt that regular succession of barbs on the collar as in the development of the normal feather is not found in absence of the rhachis. We are not prepared to say that there is no regularity but only that whatever degree of regularity there may be is subject to many exceptions.

The absence of localization of a restricted region of origin of new barb apexes, comparable to the ventral triangle in normal development, and cessation or irregularity of tangential movements, are not in themselves an explanation of the unusual behavior of the organization centers in fusing and dividing. But as they lead to crowding of the organization centers on the collar, or the reverse, they may become significant. Much-branched barbs usually have a very attenuated common base, as in Figures 14 (Pl. III) and 29 (Pl. VIII), for instance, which suggests a preceding condition of crowding of barb bases favorable to fusion. In the case of union of barb bases we do not observe such an attenuation of the bases, and this suggests the opposite condition of ample space for the bases precedent to division of the organization center.

C. THE THEORY OF REVERSED BARBS[9]

Reversed barbs occur usually in connection with a half-rhachis; they are attached opposite to the half-vane and belong in a series of tufted barbs (Pl. V). This situation at once furnishes the clue to their interpretation. We have shown that in half-vane feathers the order of origin of barbs is irregular on the defective side and that they may arise in any part of the collar. Thus they may arise at any time adjacent to the half-rhachis. If we made the assumption that the influence of the half-rhachis in attracting barbs to itself, which results in the formation of the regular half-vane, extends also in a slight degree to the defective side, a barb arising from a center close to the half-rhachis would fuse with it by its newly formed apex. This barb would then act as a block to fusion of any more remote ones on account of the rapid progress of keratinization. We shall call it the "first reversed barb" (Fig. 5, 1).

On the same assumption late-formed portions of the same barb would likewise fuse with the half-rhachis, involving thus its entire length (Fig. 5, 1), but it would not constitute an independent reversed barb. In such a case the only evidence of the first reversed barb would be a series of barbules along the defective side of the half-rhachis. Barbules are, in fact, usually found along the defective side of a half-rhachis, and there is no other source for them than from the defective side.

The first independent reversed barb (Fig. 5, 2) arises by division of the organization center of the first reversed barb, on the same principle as the origin of united barbs, thus establishing a center independent of the rhachis. According to its mode of origin, and as an observed fact, its point of attachment to the half-rhachis succeeds a series of barbules on the half-rhachis, which represent the apex of the first reversed barb; in rare cases this may reveal its barb nature by a minute free tip. Either center may divide again; in the

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[9] The theory presented here is the result of much cogitation and discussion between the authors. We believe that it is consistent with all the facts and with the theory of united barbs. An alternative theory, presupposing separate origin of each barb of a series of reversed barbs, would have to postulate the successive origin of new centers between the first reversed barbs and the half-rhachis, which is impossible as long as the first reversed barb continues to grow along the half-rhachis.

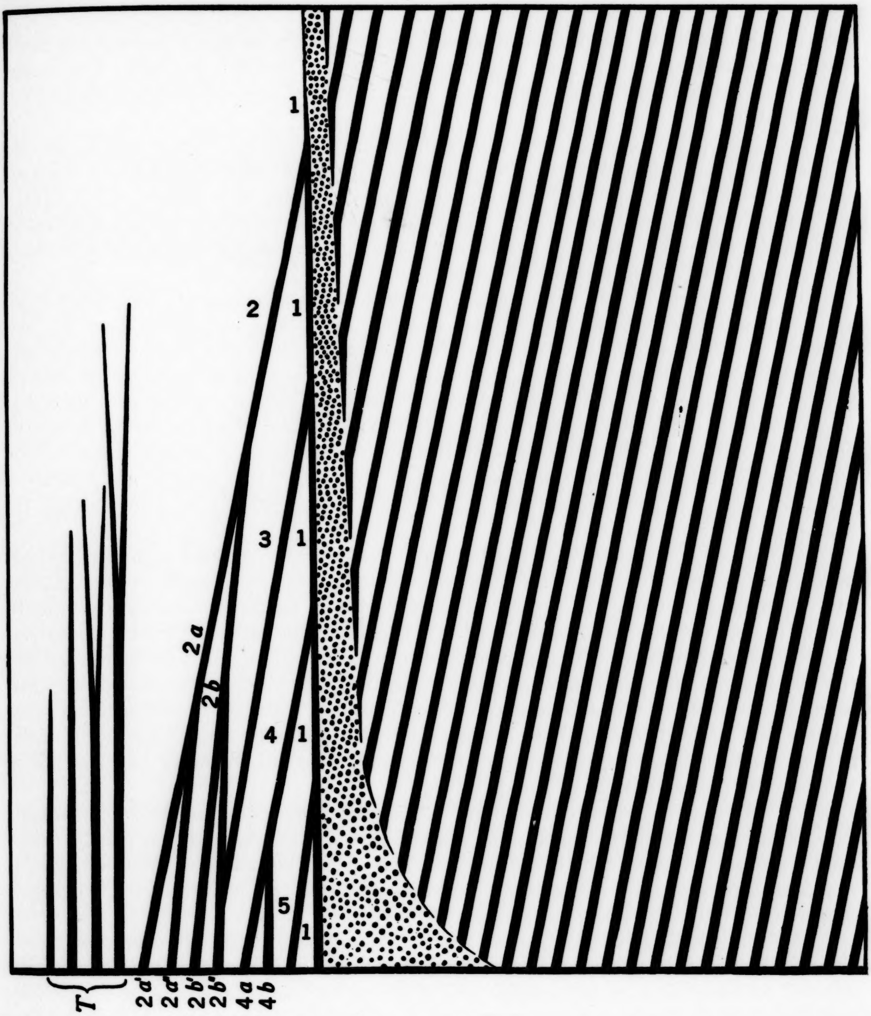

FIG. 5.—Diagram to illustrate the theory of the origin of "reversed" barbs in half-vane feathers, as stated in the text; it represents an advanced stage in the development of a series of reversed barbs, the time order of which is to be read from above downward. The line across the bottom of the diagram represents the position of the margin of the collar. The right side of the diagram is based on a total preparation of a normal feather cylinder split along one side and mounted flat with the barb ridges up. The number of barbs on this side has been reduced, and the barbules are omitted. Barb petioles are represented incompletely. The stippling represents the right lateral half of the central rhachis, which is absent on the left side. The left side of the diagram is a purely theoretical reconstruction based on half-vane feathers. To the left of the reversed barbs four "tuft barbs" are represented, two of them branched (cf. description in the text).

case of the independent barb two united barbs arise (Fig. 5, *2a* and *2b*), and in the case of the first reversed barb a second independent (Fig. 5, 3) and a fused barb. This process may be further repeated (Fig. 5, *2a′*, *2a″*, etc.). All such barbs are united apically to the half-rhachis.

This account corresponds well with the specimen shown in Figure 21 (Pl. V). But other complications may arise. Two centers of independent reversed barbs may secondarily fuse (Fig. 5, 3 with *2b″*), thus producing branching as in Figure 17 (Pl. III); or the center of the most lateral reversed barb may fuse with an adjacent single or branched barb, as also shown in the same figure. But however complex the results, the principle of origin of reversed barbs remains the same.

The most apical of the independent reversed barbs may acquire free bases in the course of development (Pl. V, Figs. 20 and 21) and stand out stiffly from the half-rhachis. It is obvious that such free barbs have ceased to grow at a rate sufficient to keep up with the axial elongation of the feather as a whole and are thus pulled loose from the collar. Most of the reversed barbs, however, maintain their connection with the collar, and thus their bases remain within the feather cylinder. A third condition (looped) is found in feathers in which the rhachis becomes bilateral at a certain distance from the apex. In this condition the base is attached on the same side of the rhachis as the apex, owing to the resumption of tangential movements on its own side (Pl. III, Fig. 17).

There is also a curious reversal of sidedness characteristic of the barbules of reversed barbs. In the vane of the normal feather the distal barbules bear hooklets, but the proximal barbules do not (Pl. II, Fig. 10). The two series of barbules differ also in other respects. In the developing barbs of the feather cylinder the side next to the rhachis normally develops the distal type of barbule. But in reversed barbs it is the side away from the half-rhachis that bears barbules predominantly of the distal type, though they may occur sporadically on the side next to the half-rhachis. This has the remarkable consequence that in half-vane feathers, whereas barbules of the proximal type occur on the barb petioles of the dominant side, on the opposite side of the same half-rhachis the barbules are predominantly of the distal type.

It is exactly as though the differential forces, which may be referred to the rhachis, that determine the sidedness of the barbules with reference to the barb, extend their influence in the case of the half-rhachis on to the defective side. There is, in any case, a reversal of the direction of action of the differential forces on the defective side.

The presumed differential action of the rhachis in determining the sidedness of barbules receives a test in the case of tuft feathers, i.e., in absence of the rhachis. The isolated barbs of such feathers carry two series of barbules above the fluff which are widely spaced, smaller than normal, and usually not differentiated. If some of them bear hooklets, they are placed indifferently on either side. But occasionally small groups of interlocking barbs occur in tuft feathers; in such cases one side of each barb bears barbules similar to the distal type of normal barbs, and the other barbules of the proximal type, and the "sidedness" is the same in all members of the interlocking group, though there is no basis of reference to denominate the rows distal and proximal. It would seem to follow that determination of distal and proximal types of barbules, though usually controlled by differential action of the rhachis, may occur without such action.

3. THE THEORY OF TANGENTIAL MOVEMENTS

Lillie and Juhn (1932) distinguished two kinds of apparent movements of barbs during their growth. Both have reference to the collar. The first is due to growth in length of the barbs, by which the distance from the apex of the barb to the collar is constantly increasing. The second is the apparent migration of the growing base of the barb along the collar from its ventral point of origin to the rhachis situated dorsally. Fraps and Juhn (1936) subsequently denominated these movements as "axial" and "tangential," respectively. The axial growth of the cylinder as a whole involves all its parts at equal rates (Lillie, 1940); hence it remains straight, and the axial growth of barbs is equal to that of the cylinder, except for the slight differential introduced by their tangential movements, in which the cylinder as a whole does not share. The position of the developing barb in the wall of the cylinder becomes a half-spiral owing to the combination of axial and tangential movements of the barbs (Lillie and Juhn, 1938, Fig. 3, diagram).

Hosker (1936) and 'Espinasse (1939) objected to the conception of tangential movements and pointed out that the conception of movement of the bases of the barbs along the collar becomes unnecessary if it is assumed that the barb-forming ridges are cut out of the collar by grooves that have a dorsal inclination; the prolongation of these grooves, as axial growth proceeds, would be progressively more dorsal in position until they met the rhachis. 'Espinasse calls this the classical theory and attributes it to Davies (1889) and Strong (1902), though on what grounds it is difficult to see. He does not suggest any extrinsic differentials determining the spiral direction of barb growth or any explanation of important exceptions, which we had previously described.

There is no question as to the translocation of the base of each barb from its point of origin, which is on one or the other side of the ventral triangle, except in the case of apical barbs, to the rhachis. There is, however, much difference of opinion as to the factors which produce translocation. Lillie and Juhn (1932) regarded it as part of a process of total concrescence of the halves of the collar in the mid-dorsal line involving the primordium of the rhachis regarded as coextensive with the barbs. A growth center for each barb was accordingly postulated in the collar. Fraps and Juhn (1936) brought cogent arguments against the idea that the primordium of the rhachis is coextensive with the complement of barbs in the collar. Lillie and Juhn (1938) accepted their idea that the primordium of the rhachis has a short, spreading base in the collar entirely independent of the barbs.

This left the theory of tangential movement of the barbs without any basis of support. Fraps and Juhn accounted for it by assuming that the dividing cells concerned are "characterized by a vectorial displacement" (1936, p. 371), without reference being made to any extrinsic differentials. They also dismissed the idea of individual growth centers of barbs as unlikely. Lillie and Juhn (1938), without any reference to vectorial displacement of dividing cells, emphasized the relation between the time of origin of the rhachis and the beginning of tangential movements and the necessity for correlation between axial growth of the shaft and the completion of tangential movements of the barbs; they also suggested a possible function of physiological gradients in the collar and the *vis a tergo* of the successive origin of new barbs at the ventral triangle as another possible contributing factor. They replaced their original theory of growth centers of barbs in the collar by the conception that the bases of formed segments of barbs act as organizers of ramogenous material arising from the collar.

It is now clear from the present experiments that the principal differential determining tangential motion of the barbs resides in the rhachis; for in absence of the rhachis tangential movements are entirely absent. Each half of the rhachis acts independently as shown by the half-vane feathers. However, the occurrence of the principal elementary abnormalities of the barbs in natural feathers when the rhachis appears entirely normal indicates clearly that slight disturbances in the regularity of tangential movements may occur independently of the rhachis in restricted loci.

The absence of tangential movements has far-reaching consequences; the barbs grow in a strictly axial orientation with reference to the cylinder; the number of barbs is greatly reduced in comparison with the normal; each barb may grow indefinitely in contrast to the normal termination of growth by attachment to the rhachis and formation of a petiole; new barbs may form apparently at any unoccupied locus in the collar, not merely in a restricted ventral locus as in the normal; division and fusion of barb centers, which are rare in normal development, become usual occurrences.

4. THE ORGANIZATION OF THE PAPILLA

The experiments demonstrate conclusively that the papilla controls the orientation and general development of the feather either directly or indirectly. However, it remains unaltered morphologically throughout the period of development of any feather and is ready to begin the regeneration of a new feather all over again whenever a feather in any stage of regeneration is plucked. This presents a problem in morphogenesis somewhat out of the usual order; though it has analogies to other cases of regeneration, there is a real difference in the discreteness of the primordium.

We shall begin by inquiring whether the control exercised by the papilla resides in its massive mesodermal component or in its delicate epidermal covering (Pl. III, Figs. 11 and 12). When we consider the fact that all the operations leave a large wounded surface which requires to be covered by proliferation and extension of the ectodermal cells and that this occurs in all the planes of the operations, it is impossible to believe that the proved prospective values that pertain to isolated portions of the papilla can proceed directly from such a flowing substratum as the epidermal covering. The repetition of defects from generation to generation of feathers from operated papillae likewise requires a more substantial basis of support. For these general reasons we proceed on the assumption that the morphogenetic control exercised by the papilla resides in the mesoderm.

It expresses itself, however, exclusively through the Malpighian layer of epidermal cells that cover its surface. These proliferate rapidly, and the external layers soon form a keratinized sheath over the germ from apex to base. Beneath this by continued multiplication of the Malpighian cells, especially toward the base, the collar becomes evident, and the barb apexes form.

The ectoderm covering the surface of the papilla is totipotent as a whole with reference to the feather, and we believe that this pertains to all its parts. Therefore, the prospective significance of these cells depends on induction by the mesoderm.

The question then becomes one concerning the time and extent of induction by the mesodermal papilla. The fact that the papilla continues to produce the pulp filling the interior of the feather cylinder, on which the nutrition of the developing feather depends, shows that the mesodermal papilla is necessary throughout development, but it does not show that the morphogenetic functions of the papilla are likewise continuous. Indeed, an experiment of Woitkewitsch demonstrates conclusively that this is not the case.

In Woitkewitsch's experiments (1936) regenerating wing coverts of pigeons of 10–13 days were pulled out as far as the mouth of the follicle and then replaced either in normal orientation or rotated 180°. Over half of eighty-three feathers, which were replaced in normal orientation, resumed growth, always in normal orientation. In one hundred and fifteen operations, in which the feathers were rotated 180° before replacement, thirty-three resumed growth and retained the reversed dorsoventral orientation. This interesting experiment shows that reversal of the collar produces reversal of subsequently developing parts of the feather, even though the papilla retains its normal orientation.

Presumably this principle goes back to the beginning of the dorsoventral organization of the collar. The first evidence of this is the arrangement of the barbs in the cylinder, which are heavy in the dorsal half and deeply pigmented in the Brown Leghorn, making a clear distinction between dorsal and ventral halves of the cylinder. The position of the rhachis is determined within this previously existing dorsal field. We are unable at present to find any earlier morphological indicators of bilateral symmetry in the ectoderm; but the induction of the dorsal field must be earlier.

We conclude that with the determination of the dorsal field the ectoderm becomes a self-differentiating system, within which secondary systems governing the mechanism of development of the feather arise. This conclusion would limit the morphogenetic functions of the papilla to induction of a bilaterally symmetrical dorsal field in the ectoderm, including, of course, the antecedent processes of multiplication of cells and of growth in the ectoderm. How is this to be reconciled with the localization of abnormalities produced in the defect experiments?

The induction of a dorsal field in the ectoderm presupposes a corresponding organization field, or organizer, in the mesoderm of the papilla. The extent of this field is clearly indicated in the experiments. The removal of the apical half of the papilla results in abnormalities in the apex of the feather; therefore, the organizer extends to the apex of the papilla. The fact that the apical half of the papilla transplanted to an empty follicle produces a small symmetrical vane which may be of considerably greater extent than the defects produced in its absence is evidence against the idea of precise localization of the axis of the feather in the papilla.

By sagittal bisection of the papilla all grades of twinning may be produced, and it is only in those cases in which the halves remain separate from the apex through the base of the papilla that completely separate twins are produced. It follows, therefore, that the organizer extends also to the base of the papilla.

We have shown that a lateral third of the papilla may induce a complete dorsal field in the ectoderm, which gives some idea of the lateral extent of the organizer; and the fact that half-vane feathers develop so frequently from ventral halves indicates that the lateral margins of the organizer must extend to the ventral half, for some portion of the organizer is necessary to explain induction of the half-rhachis.

If the induction field thus includes the dorsal half of the papilla, should we ascribe to it a mosaic organization of its own to account for localization of effects? This interpretation would produce endless difficulties if it were assumed that the elements of such a mosaic act in a time sequence parallel to the sequence of development in the feather, and it is, indeed, excluded by the results of Woitkewitsch's experiment.

We cannot be dealing with a real mosaic structure of the organizer, but at most with a field possessing gradient properties that induces a field with gradients in the ectoderm. To relate such a conception to the appearance of abnormalities having definite localization and sequence in time introduces difficulties of its own. In considering these we emphasize the fact that, as shown by the tuft feathers, the induction of a dorsal field in the ectoderm is not necessary for formation of barbs.

In the case of amputation of the apical half of the papilla we have seen that the time of emergence is at least 18 days in the case of the breast feather instead of 7 to 9 days as in the normal. If the induction of the dorsal field in the ectoderm has undergone a corresponding delay, a certain number of barbs would have formed without benefit of this field and be subject to the development of elementary abnormalities. When induction of the dorsal field in the ectoderm becomes effective and the rhachis arises, the remainder of the development of the feather will become normal, and the abnormal barbs will be carried as a defective apex. Thus the apical location of defects in this experiment does not require the assumption of corresponding localization in the organizer but can be explained by delay in the inductive action of the organizer, the duration of which determines the extent of the apical defects.

The localization problem involved in the lateral defects of half-vane feathers may also be explained on a quantitative basis: a lateral half, or even a third, of the organizer may induce a complete dorsal field in the ectoderm. But when the attempt is made to remove the dorsal half of the papilla, and hence the entire organizer, a smaller lateral margin of the organizer may be left behind in the ventral half from which half-vane feathers are derived. This lacks the power to control the organization of the entire ectoderm, and hence organized and unorganized portions lie side by side. It is a significant fact that we have in our records no case of a diminished bilateral rhachis forming a small symmetrical vane with adjoining tufted barbs, whereas we have twenty-one cases of the half-vane type. We are forced, therefore, to the conclusion that lateral margins of the dorsal organizer of the papilla induce lateral vanes only; and from the fact that these may be either right or left (ten right and eleven left in our cases) we also conclude that the right and left margins induce right and left vanes, respectively.

As material for study of morphogenesis, feathers possess the unusual, and indeed unique, advantage that the operated germ (papilla) functions in the production of successive generations. The remarkable fact is that the same defects continue to occur as in the first generation. It is difficult to reconcile the continued lack of power of regulation of the papilla thus expressed with the quantitative interpretation of localization in the dorsal organizer that we have given. Nevertheless, we believe that a quantitative theory is the best guide for further research.

POSTSCRIPT

Since the present paper was completely printed, our attention was called to T. H. Morgan's paper (1917, Inheritance of number of feathers of the fantail pigeon, Amer. Nat., **52**: 5–27), in which "split" tail feathers are described and illustrated in F_2 and back crosses between fantail and homer pigeons. Such feathers present all degrees of doubling such as we have described in our sagittal bisections of the papilla (p. 117). Morgan's general conclusion is that "we are dealing with a single rudiment that has split at a very early stage into two parts that have completed themselves as whole feathers, so far as this intimate union in the middle line of the bud permitted. There are no indications that the split feather is due to the union of two separate rudiments that have been pressed together so closely as to interfere with the full development of each when they came in contact."

The comparison furnishes a good example of a phenotypic result that may be attained in identical form by the operation of genetic factors or of somatic operations. It is interesting to note that in twenty out of twenty-four of Morgan's cases "the doubling occurs near the middle of the tail," for this suggests that a study of fantail embryos may furnish an ontogenetic explanation of the fantail condition.

LITERATURE CITED

Danforth, C. H. 1929. The effect of foreign skin on feather pattern in the common fowl (*Gallus domesticus*). Roux Arch. f. Entw'mech., **115**:242–52.

———. 1937. Pigment cells in heterogeneous feathers. Anat. Rec., **68**:461–68.

Davies, H. R. 1889. Die Entwicklung der Feder und ihre Beziehungen zu anderen Integumentgebilden. Gegenbaurs morph. Jahrb. **15**:560–649.

'Espinasse, P. G. 1939. The developmental anatomy of the Brown Leghorn breast feather, and its reactions to oestrone. Proc. Zoöl. Soc. London, A, Part IV, **109**:247–88 (published January 24, 1940).

Fraps, R., and Juhn, M. 1936. Developmental analysis in plumage. II. Plumage configurations and the mechanism of feather development. Physiol. Zoöl., **9**:319–77.

Hosker, A. 1936. Studies on the epidermal structures of birds. Phil. Trans. Roy. Soc. London, B, **226**: 143–88.

Juhn, M., and Fraps, R. 1936. Developmental analysis in plumage. I. The individual feather: methods. Physiol. Zoöl., **9**:293–317.

Kuhn, O. 1932. Entwicklungsphysiologische Untersuchungen an der Vogelfeder. Roux Arch. f. Entw'mech., **127**:456–541.

Lillie, F. R. 1940. Physiology of development of the feather. III. Growth of the mesodermal constituents and blood circulation in the pulp. Physiol. Zoöl., **13**:143–75.

Lillie, F. R., and Juhn, M. 1932. The physiology of development of feathers. I. Growth-rate and pattern in the individual feather. Physiol. Zoöl., **5**:124–84.

———. 1938. Physiology of development of the feather. II. General principles of development with special reference to the after-feather. *Ibid.*, **11**:434–48.

Strong, R. M. 1902. A case of abnormal plumage. Biol. Bull., **3**:289–94.

Willier, B. H., and Rawles, M. E. 1940. The control of feather color pattern by melanophores grafted from one embryo to another of a different breed of fowl. Physiol. Zoöl., **13**:177–99.

Woitkewitsch, A. A. 1936. Über den Ursprung des Materials bei der Bildung der Federanlagen. Roux Arch. f. Entw'mech., **135**:664–70.

PLATE I

Fig. 6.—Normal breast (*A*) and saddle (*B*) feathers of Brown Leghorn capon; dorsal view. $\times 1\frac{1}{10}$.

Fig. 7.—Second generation chimaera produced by grafting a lateral half of a papilla of the saddle tract to the contralateral half of a breast papilla in a breast follicle on the same side of the body, Brown Leghorn capon. The left side of the chimaera is from the breast, and the right side from the saddle (see p. 118); age of feather, 94 days; dorsal view. $\times 1\frac{1}{10}$.

PLATE II

Fig. 8.—Completely formed breast feather of a Brown Leghorn capon dissected after KOH treatment, showing lateral, or ramic, components of the rhachis stripped off from the central component with the whole complement of barbs *in situ;* dorsal view. Natural size.

Fig. 9.—Enlarged portion of left half-vane of a breast feather of a Brown Leghorn capon separated from the central rhachis, including parts of nine adjacent barbs forming the ramic component of the rhachis; the petioles of the two lower barbs are partly detached; cleared preparation in balsam; ventral view. $\times 20$.

Fig. 10.—Completely detached barb from the left half-vane of a dissected breast feather, Brown Leghorn capon; cleared preparation in balsam; ventral view. $\times 40$. *p.*=petiole; *d.r.*= distal row of barbules; *p.r.*=proximal row of barbules; *h.*=hooklets; *f.*=fibrous extension of the petiole; the arrow indicates the termination of the medulla of the barb.

PLATE III

Fig. 11.—Longitudinal section of an active papilla of a breast feather fixed immediately after plucking, Brown Leghorn capon; 25 days' regeneration. $\times 50$.

Fig. 12.—Longitudinal section of a resting (inactive) papilla of a breast feather fixed immediately after plucking, Brown Leghorn capon. $\times 50$.

Fig. 13.—Whole view of a breast feather from Experiment T in which the entire apical portion of a papilla was removed, Brown Leghorn hen; dorsal view; age of feather, 66 days post operation. $\times \frac{7}{8}$.

Fig. 14.—Enlarged apex of feather shown in Figure 13 showing apical defects in the form of missing barbs (right side) and branched barbs (both sides); left side dominant; dorsal view. $\times 3\frac{3}{4}$.

Fig. 15.—Enlarged apex of feather from Experiment T, White Leghorn hen, breast. Its apical defects consist of a short looped barb and branched barbs. Dorsal view; age of feather, 64 days post operation; length of entire feather, 8.5 cm. $\times 3\frac{3}{4}$.

Fig. 16.—Enlarged apex of feather from Experiment F.A.D. in which the dorsal half of the apical portion of a papilla was removed. White Leghorn cock, breast. Its apical defects consist of missing and branched barbs and a "ladder" due to repeated union of barbs. Left side dominant; dorsal view; age of feather, 66 days post operation; length of entire feather, 8.5 cm. $\times 3\frac{3}{4}$.

Fig. 17.—Enlarged apex of feather from Experiment F.A.D., Brown Leghorn hen, breast. Apical defects consist of reversed, branched, and looped barbs on the right. Note the group of branched barbs with bundling and the group consisting of one branched and one single barb attached to the reversed barbs. Dorsal view; age of feather, 51 days post operation; length of entire feather, 7.5 cm. $\times 1\frac{1}{2}$.

PLATE IV

Fig. 18.—Tuft feather from an experiment in which a large part of the basal portion of a papilla was removed, Brown Leghorn capon, breast; age of feather, 82 days post operation. Natural size.

Fig. 19.—Tuft feather completely devoid of a rhachis; from F.B.D. operation on a resting breast papilla, Brown Leghorn male; age of feather, 73 days post operation. Natural size.

PLATE I

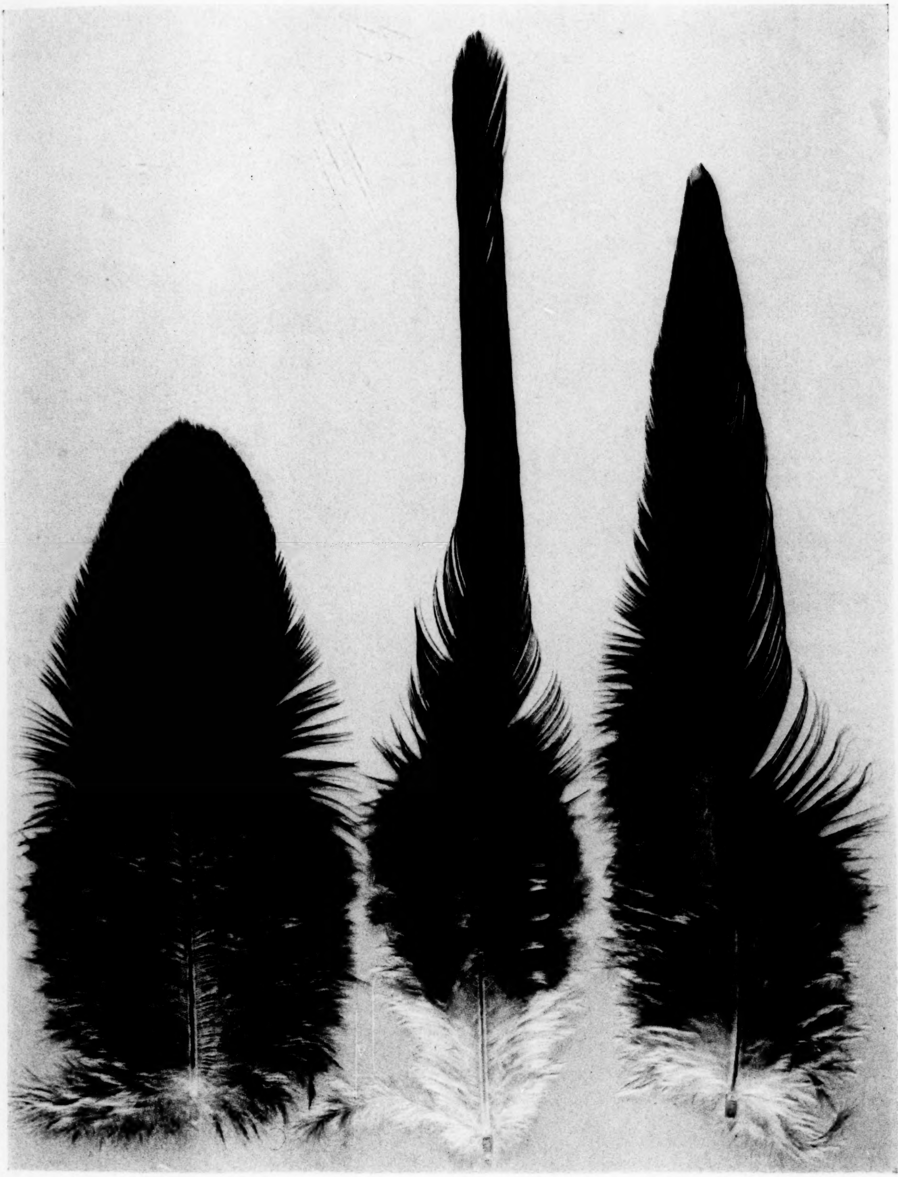

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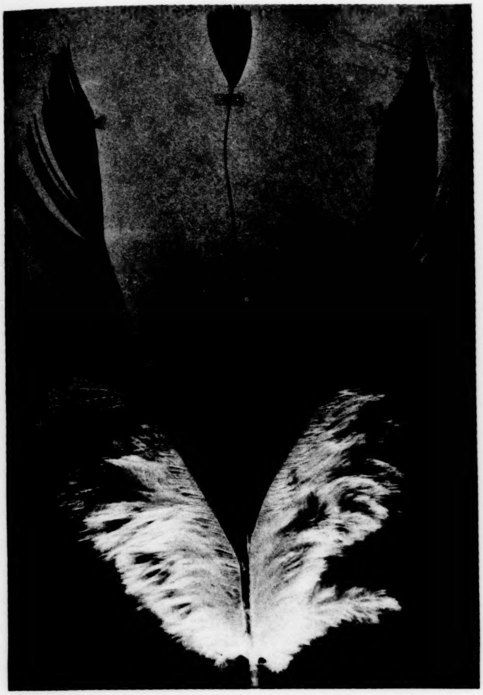
PLATE II



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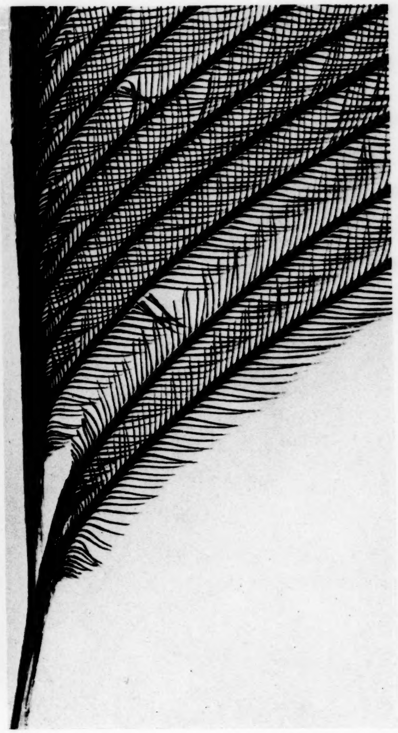

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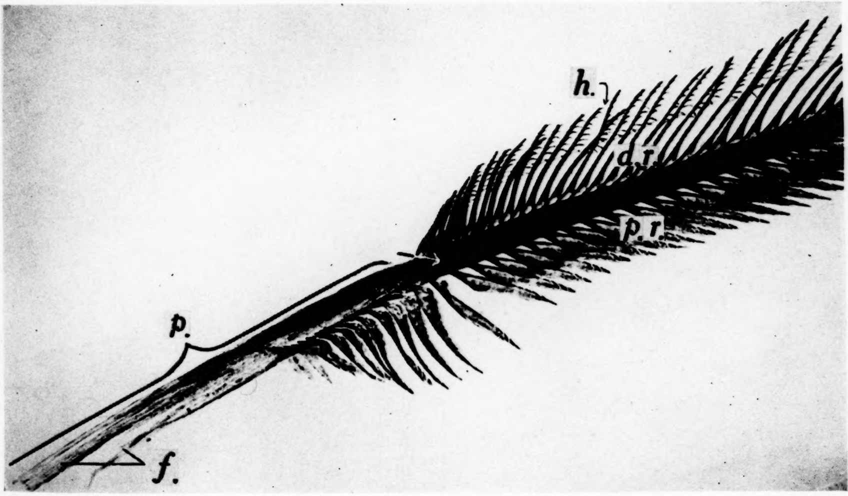

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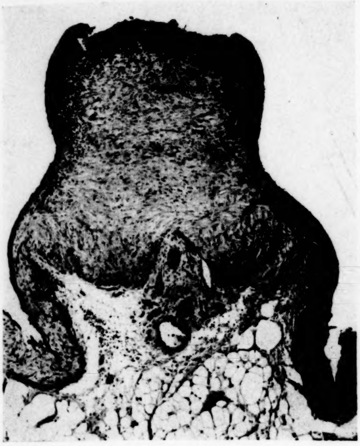
PLATE III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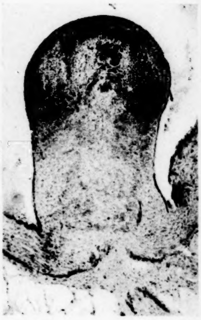
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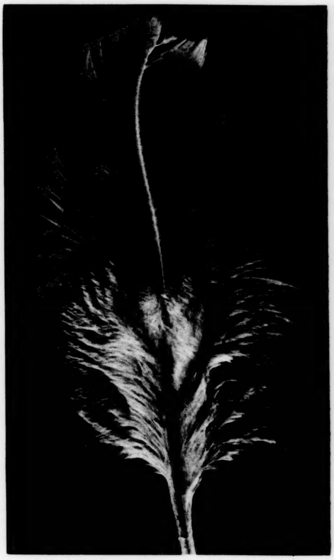
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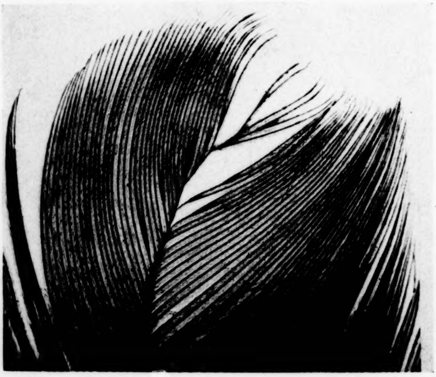
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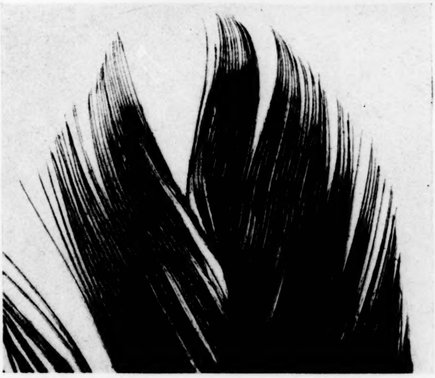
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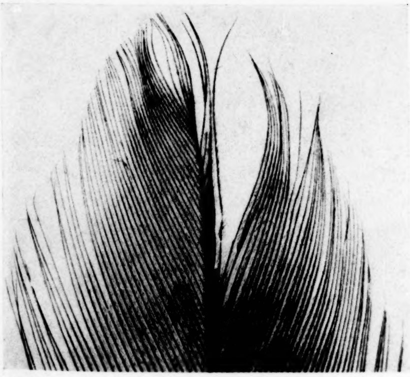
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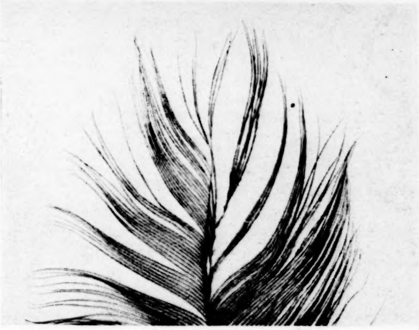
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PLATE IV

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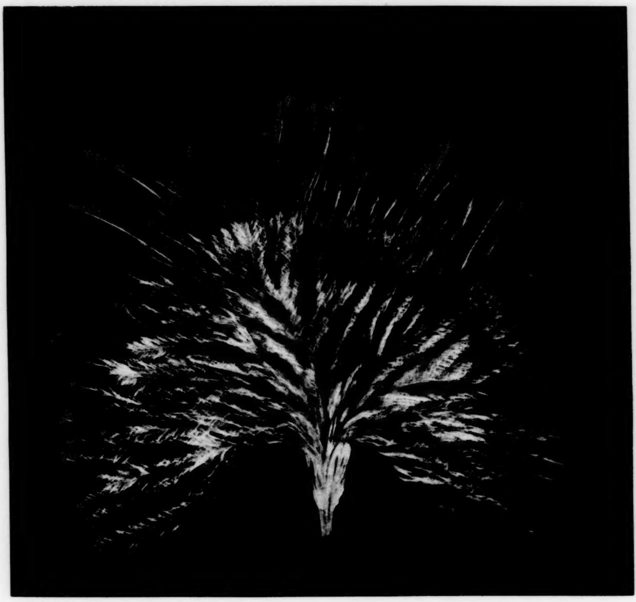

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PLATE V

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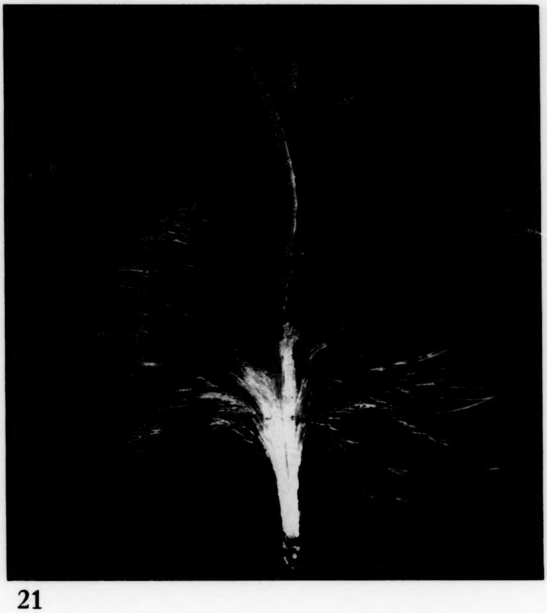

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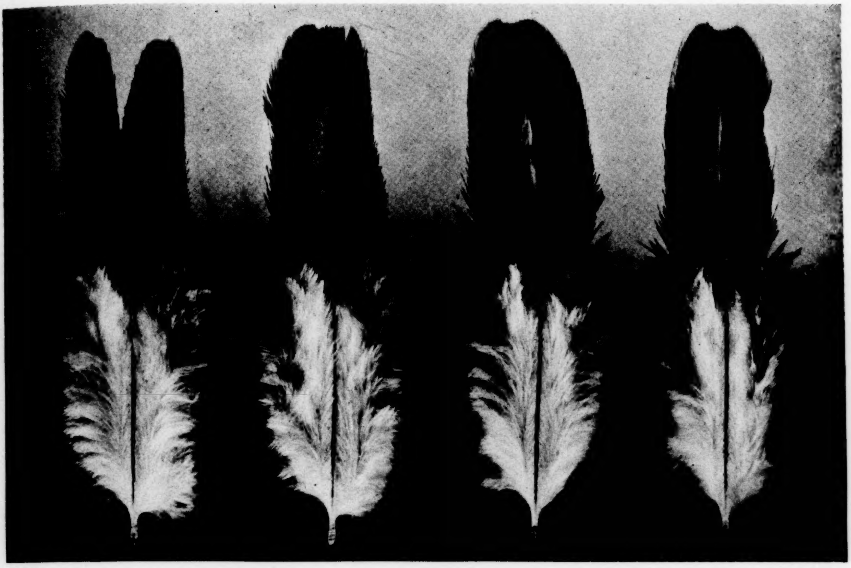
PLATE VI



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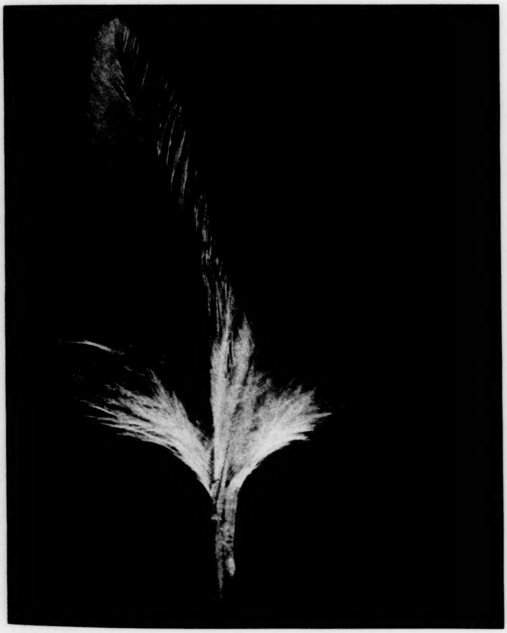

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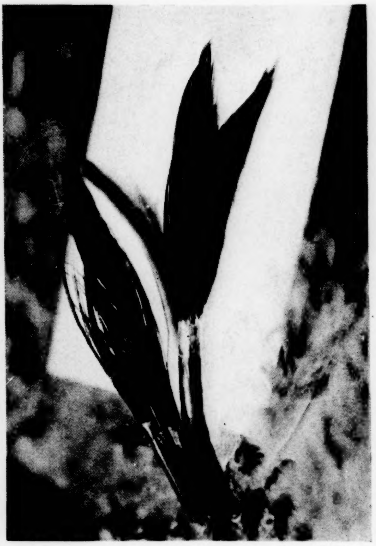

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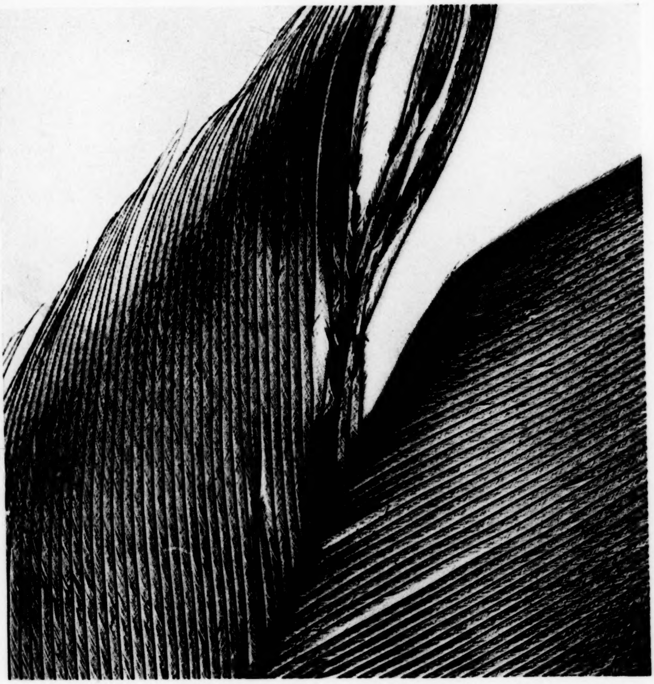
PLATE VII



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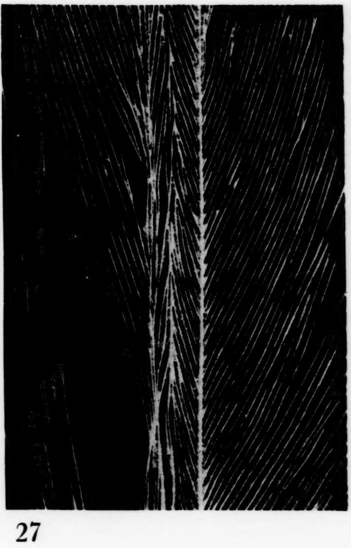

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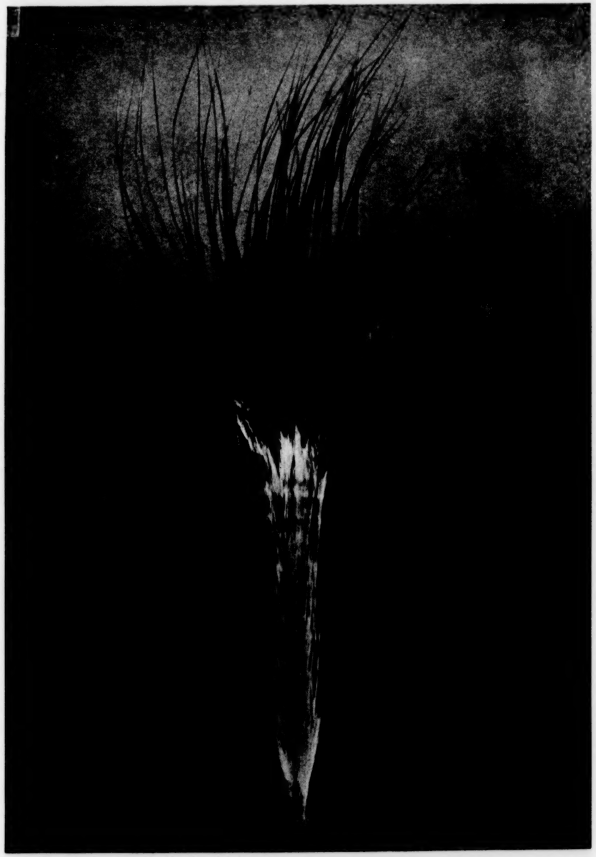
PLATE VIII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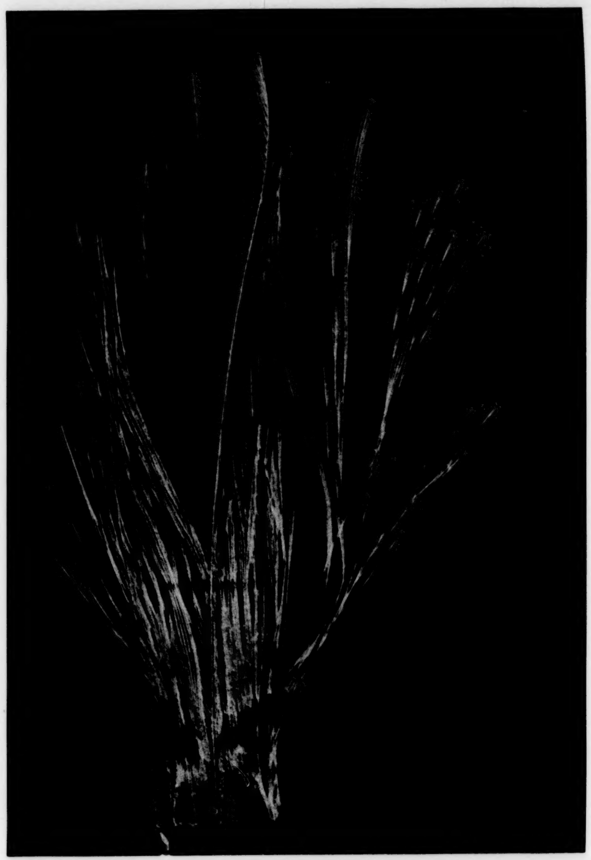
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PLATE V

FIG. 20.—First (left) and second (right) generations of a half-vane feather from Experiment F.B.D. in which the entire dorsal half of a papilla was removed, Brown Leghorn hen, breast; right side dominant; dorsal view; age of feather, 59 days for first generation, 48 days for second generation. Natural size.

FIG. 21.—Half-vane feather from Experiment F.A.D. in which the dorsal half of the apical portion of a papilla was removed, Brown Leghorn hen, breast; dorsal view; left side dominant; age of feather, 46 days post operation. Natural size.

PLATE VI

FIG. 22.—Apical twinning. Left to right: first to fourth generations from the same follicle from a sagittal isolation experiment, Brown Leghorn capon, breast. Dorsal view; age of feathers, 66 days for first generation, 72 days for second generation, 58 days for third generation, 65 days for fourth generation, post operation (see text, p. 121, for description). $\times \frac{5}{6}$.

FIG. 23.—Twin from a sagittal isolation experiment, White Leghorn hen, breast; dorsal view; age of feather, 40 days post operation. $\times 1\frac{2}{5}$.

FIG. 24.—Two pairs of twins photographed on the living bird, from a sagittal isolation experiment, Brown Leghorn capon, breast. Both pairs regenerated in a single follicle; in the one on the left each part has its own separate sheath; in the one on the right there is one common sheath for both; dorsal view; age of feather, 39 days post operation. Natural size.

PLATE VII

FIG. 25.—Apex of feather from a sagittal isolation experiment, White Leghorn hen, breast. Its apical defects consist of missing barbs, a crossing of two barbs (lower left), and bundling and branching of barbs near tip. Ventral view; age of feather, 47 days post operation; length of entire feather, 7.2 cm. ×6.

FIG. 26.—Enlarged apex of feather from Experiment T, Brown Leghorn hen, breast. It illustrates apical defects consisting of union (on the right side), branching and bundling of barbs (on the left side). Dorsal view; age of feather, 59 days post operation; length of entire feather, 8.0 cm. ×5.

FIG. 27.—Portion of a feather from Experiment T in which the entire apical portion of a papilla was removed, Brown Leghorn hen, breast. It illustrates on the left side a highly complicated pattern of abnormalities consisting of branching, union, and bundling of barbs producing simulation of a rhachis. Ventral view; half-vane at right is dominant; age of feather, 59 days post operation; length of entire feather, 5.2 cm. ×5.

PLATE VIII

FIG. 28.—Tuft feather with basal rhachis, from F.B.D. operation on a resting papilla, Brown Leghorn male (same experiment from which the feather shown in Pl. IV [Fig. 19] is derived). Dorsal view; age of feather, 73 days post operation. $\times 1\frac{1}{2}$.

FIG. 29.—Exceptional form of half-vane feather with the feather cylinder split flat, from an isolation experiment in which a breast papilla was divided into four approximately equal axial parts, White Leghorn male. Its derivation from the parts is not known. It illustrates bundling of barbs along an isochrone which extends through both the dominant and the defective half-vanes. Note slender attachments of much-branched barbs on the right. Age of feather, 40 days post operation; dorsal view. ×3.

MANIFESTATIONS OF INCOMPATIBILITY IN LIMB GRAFTS MADE BETWEEN BIRD EMBRYOS OF DIFFERENT SPECIES[1]

(Two plates, twelve figures)

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MANIFESTATIONS of host-graft incompatibility in heteroplastic transplants in Amphibia have been described by Hewitt (1934), Schwind (1937), Twitty (1937), Humphrey and Burns (1939), and others, while Loeb and other investigators have studied the problem in mammals. With the exception of several investigations made by the use of the chorio-allantoic technique, similar studies have not been reported in birds. Apparently Rawles (1938) was the first to make heteroplastic skin transplants to the body wall of avian embryos. She found on transplanting epidermis of robin embryos to the wing-bud region of embryonic chicks that the feathers produced at the site of the graft were of host type but colored like the donor. No evidence of an incompatibility was reported in this study. The author (1939*a*) described the successful transplantation of a chick limb bud to an embryonic turkey host and later made reciprocal transplants between various species of bird embryos (Eastlick, 1939*b*, 1939*c*). No macroscopic evidence of host-graft incompatibility was observed in hosts which were killed before the time of hatching. An incompatibility was observed, however, in all cases in which the hosts survived two or more weeks beyond the time of hatching. It is the object of this paper to describe macroscopic evidences of a reaction syndrome observed in duck, guinea fowl, chukar partridge, and quail appendages attached to chick hosts. For a preliminary report see Eastlick (1939*d*).

MATERIAL AND METHODS

The chicken, chukar partridge, and quail eggs were obtained at the poultry farms of the University of Missouri,[3] while the guinea-fowl and duck eggs were obtained from various hatcheries. White Leghorn and Barred Plymouth Rock chick embryos were used as hosts; the ducks were Wild Mallard and White Pekin, and the guinea fowls were of the Pearl variety.

Donor and host embryos usually were $2\frac{1}{2}$–$3\frac{1}{2}$ days old (26–38 somites), although some younger and a few older stages were used. The method of making the transplant was a modification of the method developed by Hamburger (1933, 1938). With the removal of both the wing and the posterior limb buds four grafts could be made from a

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[1] Aided by a grant from the Research Council of the University of Missouri and the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2] National Research Council Fellow at the University of Chicago. The author acknowledges courtesies of the Department of Zoölogy and advice of Dr. Paul Weiss in the preparation of the manuscript.

[3] The author wishes to thank Mr. James Forward, superintendent of the poultry farms at the University of Missouri, for supplying the game-bird eggs and rendering many other favors.

single donor embryo. Limb buds left in warm, sterile physiological saline solution for an hour or more were not injured, since the fourth transplant made from a donor seemed as viable as the first. Most of the transplants were placed on the host in normal orientation, although in some cases the anteroposterior or the dorsoventral axis was reversed.

GENERAL RESULTS

Considerable difficulty was encountered in getting the hosts to survive the latter part of the incubation period. This high mortality between 19 and 21 days of incubation seems to be associated with the failure of the yolk sac to retract properly owing to malposition of the host and the membranes sticking to the shell. A few of the embryos which hatched with the yolk sac incompletely retracted were saved by tucking the ectopic sac into the coelom and suturing the umbilicus.

The grafts, for the most part, were attached to the right flank of the host just ventral and posterior to the right wing, although two cases were secured in which the transplant became fused to the wing. Also in two additional hosts the right wing was suppressed completely, and the transplant seemed to have taken its place.

The extent of attachment between graft and host varied greatly. Occasionally, when the entire proximal portion of the graft was fused to the host, a considerable area of the surrounding tissue seemed to be of donor origin as diagnosed by the difference in color of donor and host skin. In other instances a rather narrow bridge of tissue held the graft in place and a small area at the base seemed to be of donor origin.

THE INCOMPATIBILITY REACTION

I. DUCK GRAFTS

Of the fifteen cases secured, four bore well-formed duck legs, two had normal-shaped wing grafts, and the others had atypical wings. The grafts appeared healthy for a period ranging from 10 to 14 days after the hatching of the host, during which time a well-formed leg graft grew approximately twice as fast as the host's own leg (Fig. 1). This seems to be in accordance with the normally faster growth rate of the duck. The first indication of incompatibility in a transplanted leg was a slight swelling of the toes and the web. The edema then proceeded proximad over the graft, and within a day or more the basal portion had enlarged noticeably. The swelling continued so that within a few days the web became several times its normal thickness (Fig. 3). The skin of the graft soon was so tightly stretched that it took on a glossy appearance and ruptured in a number of places. Localized hemorrhages occurred. The epidermis at the edges of the cracks frequently thickened, forming a scab. Some sloughing of the epidermis was observed, especially on the web.

The feathers of the graft were injured during the period of swelling, for they ceased growing, became necrotic, and fell from their follicles, first in spots, later throughout the graft (Figs. 3, 4, and 5). The rest of the graft kept on growing rapidly. Its distal portion usually dragged on the floor of the cage, and the host stepped on it. Mash and debris clung to the moist surface of the leg and, within a week after the onset of the reaction, the foot and shank became covered with a heavy incrustration composed of scab and extraneous matter. Although pieces of the crust 1 cm. square or larger occasionally sloughed with exposure of the phalanges or bones of the shank, a scab was reformed, and healing occurred. Regressive changes took place in the ankle joints of the transplants; they gradually underwent ankylosis and eventually became entirely immobilized. The knee joints, however, remained movable throughout the life of the grafts.

Meanwhile, the donor tissue connecting the graft with the host also passed through a series of necrotic changes. Ordinarily, the place of fusion between graft and host could be seen clearly, since the duck skin had a yellowish appearance while the chick skin was greyish or white. At the beginning of the reaction in this area the donor skin became edematous. Some blisters developed, filled with fluid, and ruptured. Considerable areas of the epidermis sloughed, exposing the underlying dermis. The peripheral blood vessels in the area enlarged, and frequently fluid escaped. In some cases a sticky, yellow exudate formed, covering the skin around the base of the graft. The feathers within the affected area became dry and brittle and frequently fell from their follicles, but in no case did the reaction spread beyond the limits of the donor tissue. The grafts passed through the most drastic period within four weeks and by the end of this time were covered, entirely or in part, with a scab (Figs. 4 and 5). Healing then followed over a period of several weeks (Figs. 2 and 6). The final result of this phase was variable as illustrated by the following case histories.

Host DC 27 possessed a well-formed Mallard leg with the exception of a missing fourth toe (Fig. 1). Healing took place on the proximal portion of the transplant and gradually extended distally. The terminal end of the foot bore a scab during the eighth week (Fig. 2), but at the end of the ninth week the graft was completely healed. During the course of healing an abundance of scar tissue was formed between the toes so that the web was obliterated and a club-shaped foot resulted. At the time of autopsy in the thirteenth month, this graft still appeared healthy.

Host DC 111 possessed a wing graft which became completely covered with a scab. As this began to recede during the fifth week, the graft started to grow excessively; and at ten weeks, when the host died of coccidiosis, the wing was 33 mm. wide at the elbow and apparently had not yet reached its maximum size.

DC 129 (Fig. 5) bore a slightly atypical leg which was healing up to the seventh week when the host amputated the foot. The remaining portion of the leg then began to decrease in size, and when the bird was sacrificed in the sixteenth week the graft had almost disappeared.

DC 125 bore a leg from which the foot was missing. The graft underwent the usual reaction; but recovery occurred, and at the eighth week only the terminal end of the shank bore a scab. Host feathers grew down over the proximal portion of the leg from the base of the graft. The transplant appeared healthy up to the twelfth week, when it underwent a second reaction at which time intense swelling occurred in the distal end followed by edema in the entire leg. The skin became so tightly stretched that it ruptured. Considerable loss of fluids occurred, which matted the feathers. When they were clipped loose, the skin sloughed in sheets with the exertion of a slight tension. The surface of the leg became greenish in color, and a foul odor emanated from the graft. Fibrosis seemed to occur, as the graft was hard to the touch. By the fifteenth week the graft was decreasing in size owing to resorption and was also being cut free from the body of the host by a constriction. Since it appeared that the transplant would soon be severed, the host was sacrificed and tissue fixed for histological studies.

The fate of a duck graft following the incompatibility reaction seems to be very diverse, for it may (1) heal, cease growing, and persist as a small appendage; (2) grow abnormally large; (3) be resorbed; or (4) recover temporarily and then undergo a second reaction in which it is destroyed.

None of the hosts died or became seriously ill as a consequence of the incompatibility reaction. Well-developed grafts did cause the hosts considerable difficulty in locomotion, and in those instances in which the grafts underwent a second reaction the hosts were distinctly uncomfortable, a droopy appearance was assumed, and the furnishings of the head lost color.

II. CHUKAR PARTRIDGE GRAFTS

Six hosts hatched and reached maturity. Three bore well-formed leg grafts; one had a normal-shaped wing, while the other two possessed atypical wings. The incompatibility reaction occurred between 9 and 14 days after hatching, the onset being on the ninth day in two cases, the eleventh in one, and the fourteenth day in the others.

Two cases which illustrate the reaction syndrome will be described. Host X 29 bore a well-formed leg attached under the right wing and carried so that the host could not step on it. The feathers of the transplant were only partially colored, apparently because of the fact that many of the graft melanophores had migrated into the right wing of the host (Fig. 7). The region of fusion of graft and host tissues was indicated clearly by the dark color of the chukar partridge skin. On the ninth day the scales of the toes were dry, and some desquamation took place (Fig. 7), although no intense reaction occurred until the fourteenth day when the skin became edematous. Rupture of the skin at the ankle and at other places was followed by extravasation of fluids. Irregular scabs then formed on the distal portions of the leg and foot (Fig. 8). The graft increased in size for four weeks (Fig. 8), then was slowly resorbed; the destruction became complete when the host was 4½ months old.

The feathers, seemingly of donor origin, at the base of the graft became matted in the first few days of the reaction. They were unable to survive, and by the seventh week the graft was entirely bare. During the eighth week host feathers began to appear on the denuded regions. The new feathers were uncolored, although the original ones had been pigmented. Apparently the chukar melanophores located in the donor skin were destroyed during the tissue reaction. On the other hand, the chukar pigment cells which had migrated into the wing of the host were not injured, since they continued to supply melanin to the wing feathers of the host.

Host X 69 bore a well-formed chukar partridge wing in place of the right wing of the host. The onset of the reaction occurred on the fourteenth day. The wing became very swollen, the skin ruptured, and rather severe hemorrhages took place. The primary feathers, which had grown up to this time, became swollen at their bases and soon dried (Fig. 9). Within a week the entire wing was necrotic and was gradually pinched off at its base.

Apparently these two grafts were eliminated by quite different procedures; in one case the graft was slowly resorbed, while the other underwent necrosis and was severed from the host by a progressive constriction at the base of the transplant.

III. QUAIL GRAFTS

Of the seven cases secured, four possessed normal legs, and the others had atypical wing grafts. The grafts became slightly edematous 7–10 days after hatching, and necrosis soon followed. The rate of breakdown was rapid, and the grafts were soon eliminated. In other respects the syndrome in quail grafts seemed to parallel that observed in chukar partridge transplants.

IV. GUINEA-FOWL GRAFTS

Nine hosts were secured, five with leg and four with wing grafts. Four of the leg transplants were of normal shape, while the rest of the transplants were atypical. The onset of the reaction occurred soon after hatching, being at one day in one instance, two days in another, and on the fifth day in the rest. The first indication of the reaction was a slight swelling of the toes soon followed by edema throughout the graft. Within a day after the onset the toes began to turn black, to shrivel, and the foot fell off (Fig. 10). Next the region just proximal to the terminal end became necrotic, constricted, and broke off (Fig. 11). In this way some grafts became reduced in length until only a basal portion remained (Fig. 12). At a later date even this was lost by a constriction at the junction between host and transplant.

In some instances the entire graft became shriveled within a week after the onset of the reaction. Transplants of this type seemed to lose their blood supply and degenerated *in toto*. The feathers on the graft and skin immediately surrounding the point of attachment appeared dry and unhealthy from the time of hatching and soon fell from their follicles. The guinea-fowl skin at the base of the graft became dry and scaly, and large flakes were shed until all the donor epidermis was eliminated or replaced by host skin. Guinea-fowl grafts seldom became so much swollen that the skin ruptured, consequently there was little extravasation of plasma or hemorrhage. Moreover, these grafts were never observed to develop scabs or to heal.

DISCUSSION

The results show that limb-bud transplants may be made successfully between embryos of different species, even between embryos belonging to different orders. The grafts may undergo normal morphogenesis and appear healthy during the embryonic period, but evidence of an incompatibility occurs in varying periods after hatching. The onset of the reaction seems to occur at a definite time in each species: the interval between hatching and the reaction is 10–14 days in duck and chukar partridge grafts, 7–10 days in quail grafts, and around 5 days in guinea-fowl grafts. The rapidity of breakdown of the transplants follows a similar order, that is, the guinea-fowl grafts were eliminated in the shortest time.

While the reactions vary in detail in the different species and even in individual cases within a given group, the syndrome seems to affect commonly the blood vessels and the skin. There was an initial increased supply of blood to the affected regions as indicated by the dilation of the cutaneous vessels and the swelling of the digits. Stasis followed by hemorrhage and extravasation occurred in all cases, but to a much less extent in guinea-fowl grafts. Rupture of the peripheral capillary bed was indicated by the presence of discolored sinus-like areas in the dermis. The skin was seriously affected as indicated by edema, discoloration, scaling, and sloughing, followed by the formation of a heavy scab in all except guinea-fowl grafts. The feathers dried, became brittle, and fell from their follicles. In all cases except one of the duck grafts the final step was the destruction and elimination of the transplants. In many respects the reactions seem to resemble rather closely those found by Humphrey and Burns (1939) in heteroplastic grafts or parabiosis in Amphibia. One notable exception is that many of the amphibians died during the reaction, while none of the birds succumbed.

A number of investigators have observed the breakdown of homoplastic transplants made between birds. Roxas (1926) transplanted Leghorn testes into Sebright capons and secured a 60 per cent "take," while the reciprocal grafts were only 8 per cent successful and many of the hosts died. Juhn (1927) found that the Sebright serum seemed to hemolyze Leghorn corpuscles to a greater extent than the reverse. She considered it likely that a toxin produced by a breakdown of the Sebright tissues killed the Leghorn hosts. Danforth and Foster (1929) and Kozelka in a series of papers (1929–33) reported that a high percentage of homoplastic integumental grafts made between hatched chicks were resorbed or destroyed within 2–12 weeks after the operation. In fact, Kozelka (1929) reported that 74 per cent of his homografts were resorbed within 2 months. He observed various phases in the breakdown process, e.g., excessive vascularization, swelling, and occasional sloughing of the skin in parts of the body quite far removed from the graft. The reactions described by Kozelka do not seem as severe as those noted in the present study. This may be due in part to the larger size attained by the limb grafts and to the more distant relationship between donor and host. Kozelka (1929) found no evidence of reaction in autografts and in 1932 showed that more successful takes occurred in grafts made between closely related birds than when the relationship was distant. Loeb and Wright (1927) have suggested that the incompatibility reaction is correlated with the number of strange genes in the transplant. The author has noted evidence (unpublished data) of host-graft incompatibility in homoplastic limb transplants in the fowl. In the first group of experiments these reactions occurred during the fifth month after hatching, while in the second they began in the seventh week. The eggs in the two series were secured from different hatcheries; also they were not of the same varieties. The reaction occurred in only a few of the hosts and seemed to be somewhat different from that observed in heteroplastic transplants, although more cases will have to be secured before any general statement concerning the nature of the differences can be made.

Holman (1924) observed an intense dermatitis and desquamation following the grafting of skin of a mother to her child. He described the reaction as "similar but not identical to anaphylaxis." Recently Sandstrom and his co-workers (1939) described a fatal anaphylactoid reaction produced by transplanting macerated nephritic tissue of hatched ducklings to the chorio-allantoic membranes of embryonic chicks. The reaction was not produced by grafts of intact metanephros of hatched ducklings or by explants of embryonic tissue. Sandstrom is of the opinion that a species-specific factor of a toxic nature appears at the time of hatching and is liberated by maceration. This explanation seems plausible, since Murphy (1914) found that chick embryos became intolerant to foreign tissues between the eighteenth and the twenty-first day of embryonic life. If bird embryos do develop species specificity slightly before or at the time of hatching, why do heteroplastic grafts fail to show evidence of incompatibility immediately after hatching? A number of possibilities may be mentioned. (1) The graft may not produce incompatible proteins or toxic substances until after the hatching of the host. (2) Toxic substances may be produced, but the quantities may be insufficient to call forth the reaction. (3) The protective mechanisms of the host may not be sufficiently well developed to combat the foreign tissues until a latent period has passed. (4) The toxic substances may not be released to any extent from intact cells, and hence the reaction may be delayed until the breakdown is once initiated. (5) Grafts and host tissues may have become more or less "adjusted" to one another during the embryonic period, and this tolerance may not be lost at once. For example, it is possible that a duck limb when grown on a chick host may not possess the same degree of "duck specificity" that it would have had under normal conditions of development. The fact that the graft is isolated from other duck tissues, is nourished by metabolic products of the chick host, is in intimate contact with the host tissues, body fluids, etc., from the time when both donor and host tissues are embryonic may lead to a weakening of the tissue specificity factors in the graft. (6) The physiological age of the grafts may not be identical with their chronological age, since the transplants may be retarded because of their foreign environment. Guinea-fowl and duck embryos hatch in 28 days, while chicks hatch in 21. When this fact is taken into consideration it is obvious that the incompatibility reaction generally begins when the guinea-fowl graft is 26 days of age, or 2 days before the term of hatching. The onset of the reaction in duck grafts corresponds to a 5–7-day duckling. (7) External evidence of the incompatibility reaction may not indicate reliably the time of appearance of antibodies in the host. For example, the host may produce antibodies against duck grafts from the time of hatching, but the tissues of the transplant may be resistant and fail to show marked changes until 10–14 days later. On the other hand, guinea-fowl tissues may be more sensitive and consequently break down soon after the hatching of the host.

Hewitt (1934) has suggested that the failure of a heterograft to survive in amphibians may be due to (1) inability to use the food supplied by the host, (2) inability to excrete waste products, (3) discrepancies between the rate of growth of graft and host tissues, etc. These factors may be involved in the breakdown of heteroplastic grafts between birds.

It seems strange that a limb graft from a duck—a species which belongs to an entirely different order from that of the chick—is able to survive, while limbs from species within the same order as that of the host are destroyed. It is possible that the duck grafts are largely replaced by host tissues, while this does not occur in the other species.

Although it has not been demonstrated experimentally, it seems logical to assume that the foreign proteins of the heteroplastic grafts sensitize the hosts so that defense mechanisms are brought into play. The host may respond by walling off the graft with connective tissue, by mass infiltration of white blood cells, by the production of immune bodies, or by all three. A study will be made in an attempt to find whether or not immune reactions can be correlated with the incompatibility syndrome in heteroplastic transplants made between bird embryos.

SUMMARY AND CONCLUSIONS

1. Duck, chukar partridge, quail, and guinea-fowl limb buds were transplanted to embryonic chick hosts. Thirty-seven of the chicks hatched, and many were raised to maturity.

2. The grafts underwent an incompatibility reaction within a rather definite time after the hatching of the hosts. The time of appearance of the syndrome and its severity seem to vary in each of the species. One of the duck grafts recovered from the reaction and persisted for 13 months, while other duck appendages survived for lesser periods of time. The chukar partridge, quail, and guinea-fowl transplants did not heal following the incompatibility reaction and subsequently were eliminated.

3. The reaction seems to affect seriously the vascular system of the graft as indicated by stasis and edema. Also the donor skin ruptures, sloughs, and seems to be destroyed.
4. Probable factors underlying the syndrome are discussed.

LITERATURE CITED

Danforth, C. H., and Foster, F. 1929. Skin transplantation as a means of studying genetic and endocrine factors in the fowl. Jour. Exper. Zoöl., **52**:443–70.

Eastlick, H. L. 1939*a*. The morphogenesis of a hind limb of a 60-hour chick transplanted to a turkey embryo. Anat. Rec., Suppl., **73**:17.

——. 1939*b*. Reciprocal heterotransplantation of limb primordia between duck, turkey, guinea, and chick embryos. Nature, **144**:380–81.

——. 1939*c*. A study of feather character in limbs transplanted between embryos of different bird species. Proc. Natl. Acad. Sci., **25**:551–57.

——. 1939*d*. Observations on species differentials in birds as studied by means of heterotransplantation of embryonic limb buds. Anat. Rec., Suppl., **75**:68.

Hamburger, V. 1933. A method of transplanting limb buds in chick embryos. Anat. Rec., Suppl., **55**:58.

——. 1938. Morphogenetic and axial self-differentiation of transplanted limb primordia of 2-day chick embryos. Jour. Exper. Zoöl., **77**:379–98.

Hewitt, D. C. 1934. Xenoplastic transplantation of amphibian eye rudiments. Jour. Exper. Zoöl., **69**:235–67.

Holman, E. 1924. Protein sensitization in isoskin grafting. Surg., Gyn., and Obst., **38**:100–106.

Humphrey, R. R., and Burns, R. K., Jr. 1939. An incompatibility manifested in heteroplastic parabiosis or grafting in Amblystoma due to a toxin of cutaneous origin. Jour. Exper. Zoöl., **81**:1–42.

Juhn, M. 1927. Note on the hemolytic action of Sebright serum on Leghorn corpuscles. Biol. Bull., **52**:474–79.

Kozelka, A. W. 1929. Integumental grafting in domestic fowl; transplants of combs, spurs, and feathers in study of sex dimorphism. Jour. Hered., **20**:3–14.

——. 1931. Genetic and physiological factors involved in the absorption of integumental grafts in the Leghorn fowl. Anat. Rec., Supp., **48**:24.

——. 1932. Integumental grafting as a means of analyzing the factors determining the secondary sexual characters of the Leghorn fowl. Jour. Exper. Zoöl., **61**:431–95.

——. 1933. Serological studies of tissue antagonism in the domestic fowl. Physiol. Zoöl., **6**:159–84.

Loeb, Leo, and Wright, S. 1927. Transplantation and individuality differentials in strains of inbred rats. Amer. Jour. Path., **3**:143–67.

Murphy, J. B. 1914. Studies in tissue specificity. III. Factors of resistance to heteroplastic tissue-grafting. Jour. Exper. Med., **19**:513–22.

Rawles, M. E. 1938. The production of robin pigment in White Leghorn feather cells by skin ectoderm grafts of the robin embryo. Anat. Rec., Suppl., **72**:68.

Roxas, H. A. 1926. Gonad cross-transplantation in Sebright and Leghorn fowls. Jour. Exper. Zoöl., **46**:63–119.

Sandstrom, C. J.; Eisen, H. N.; and Siffert, R. S. 1939. Reciprocal heteroplastic chorio-allantoic transplantations of macerated chick and duck kidney tissue. Anat. Rec. Suppl., **75**:120–21.

Schwind, J. L. 1937. Tissue reactions after homoplastic and heteroplastic transplantation of eyes in the anuran amphibia. Jour. Exper. Zoöl., **77**:87–108.

Twitty, V. C. 1937. Experiments on the phenomenon of paralysis produced by a toxin occurring in Triturus embryos. Jour. Exper. Zoöl., **76**:67–104.

PLATE I

FIG. 1.—14-day White Leghorn host bearing a Mallard duck graft. Down on graft unpigmented owing to exclusion of neural-crest cells. Leg well formed with exception of missing fourth toe.

FIG. 2.—Same host as above. 8 weeks of age. Graft has healed with exception of distal end of foot. Foot is club shaped owing to scar tissue.

FIG. 3.—14-day Barred Plymouth Rock host bearing White Pekin duck leg. Host melanophores have pigmented the down on the transplant. Foot edematous, and down is becoming necrotic.

FIG. 4.—Same donor as above. Host 26 days old. Note the thick incrustation on foot and shank. The feathers have sloughed in some areas.

FIG. 5.—28-day Barred Plymouth Rock host bearing a White Pekin duck leg. Entire graft is incased in scab. Graft denuded.

FIG. 6.—Same host as in Figures 3 and 4. Host died of coccidiosis at 6 weeks of age. Note that upper portion of graft has healed.

PLATE II

FIG. 7.—9-day White Leghorn host bearing a chukar partridge leg. Donor melanophores have migrated into wing of host. Some slight desquamation of foot epidermis can be seen.

FIG. 8.—Same host as above at 28 days of age. Note the scab on foot and shank and the matted feathers surrounding the graft. Graft has reached its greatest size. Resorption took place from this time.

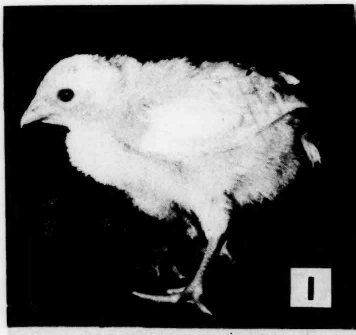
FIG. 9.—15-day White Leghorn host bearing a chukar partridge wing. Host wing suppressed. Graft necrotic. Was pinched off at base within a few weeks.

FIG. 10.—5-day White Leghorn host bearing a guinea-fowl leg. Toes of graft shriveled and necrotic.

FIG. 11.—Same host as above at 21 days of age. Foot missing. Rest of graft becoming necrotic.

FIG. 12.—Same host as above, but at 8 weeks. Entire graft "mummified." Was pinched off at base during fifteenth week.

PLATE I



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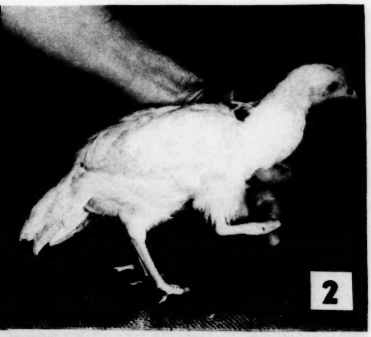

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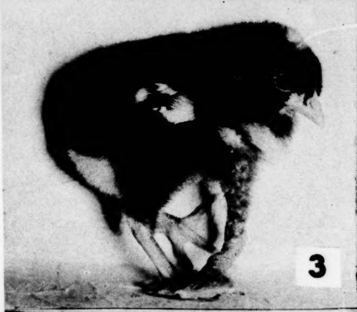

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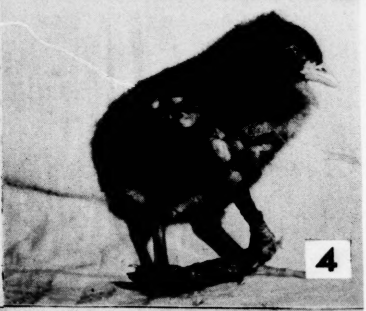

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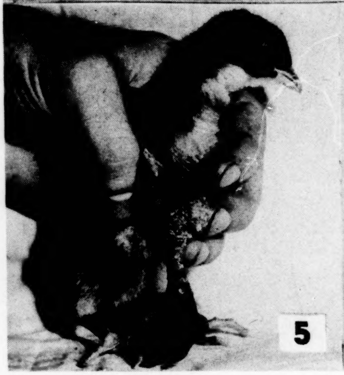

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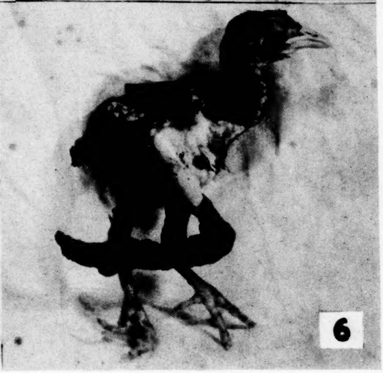

6

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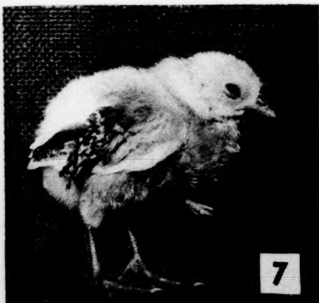
PLATE II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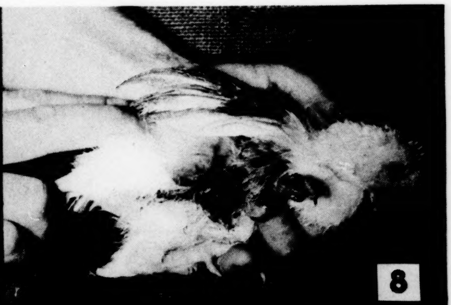
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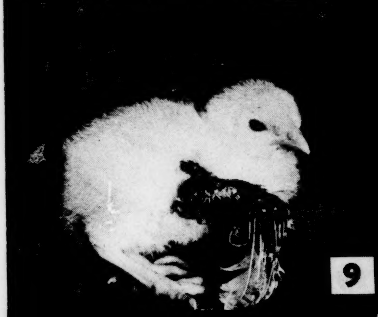
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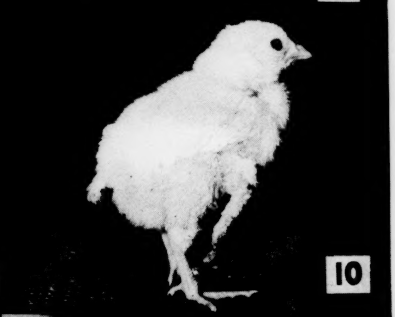
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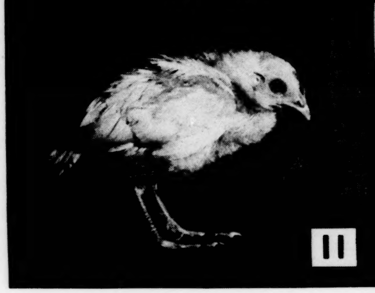
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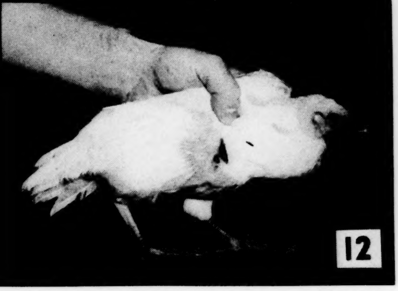
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RAT PROSTATE AND SEMINAL VESICLE GRAFTS IN RELATION TO THE AGE AND SEX OF THE HOSTS[1]

(Two plates)

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A STUDY (Price, 1936) of the results of early prepuberal castration of rats in relation to ventral prostate and seminal vesicle gland development introduced a number of problems. It was found that when rats were castrated within a few days after birth the ventral prostate continued growth, although at a slower rate, and that differentiation proceeded and the epithelium of the gland developed cell "light areas" when the rats were 17 days of age, as compared with 12 days for normal controls. These distinctive areas in the cells have been found to be constantly present in the adult functional gland, and they disappear within a few days after castration of adults. In the young castrate the light areas developed, the acini became distended, and a nearly normal histological condition was maintained until the animal was 30–40 days of age. At that time the light areas disappeared, the epithelium became flat, acini small, and the tissue typically castrate.

These findings, i.e., that the ventral prostate of the young castrated animal reached a differentiated state and maintained it for a certain limited period of time, required further analysis. The assumption was that the prostate in the young castrate differed in its behavior from that of adult castrates because of (1) some factor or factors present in the young animal and able to influence the prostate but absent in the adult or (2) an ability of the prostate to "self-differentiate." The tissue-transplantation method was used as a means of studying the problem and gaining a further understanding through prostate-transplant reactions of the hormones circulating normally in animals at various stages in the reproductive cycle. The prostate gland is well adapted as an indicator of androgenic substances because of its sensitivity, while the seminal vesicles have a higher threshold. In earlier work (Wiesner, 1934; Price, 1936) it was found that the seminal vesicles did not differentiate after early prepuberal castration but continued to grow, though to only a very limited extent. In the present study it was of interest to transplant seminal vesicles for comparison with the prostate. A preliminary report on a portion of this work was made (Price, 1937).

MATERIAL AND METHODS

Ventral prostate and seminal vesicle tissue from rats varying from 5 to over 100 days of age was transplanted into hosts ranging from 1 to 144 days of age. The transplants remained in the hosts for periods varying from 13 to 246 days. The hosts were young and old normal and castrated males, young and old normal and spayed females, and pregnant females. In all, 890 transplants were made into 316 hosts, and 335 grafts were recovered.

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[1] This work was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

TABLE 1

SUMMARY OF PROSTATE TRANSPLANTATIONS IN RELATION TO HOST AGE AT AUTOPSY

DONOR	HOST			TRANSPLANTATIONS										
Age in Days at Transpl.	Sex	Age in Days		No. Made	No. Rec.	% Rec.	Length of Transpl. Period	Age in Days	Histology					
		At Transpl.	At Autopsy						L.A.	Cast.	Un-dev.	Deg.	No Gl.	% +
Under 35 . . .	♂	Under 35	Under 35	18	10	55	13–29	25–54	8	....	....	1	1	80
			Over 35	8	2	25	23	33	2	....	....	....	...	100
	♂̸	Under 35	Under 35	37	17	46	13–20	25–54	7	2	1	5	2	41
			Over 35	18	8	44	21–123	33–131	....	5	....	1	2	0
	♀	Under 35	Under 35	12	9	75	13–20	25–31	6	....	....	2	1	66
			Over 35	20	13	65	23–90	33–102	1	10	....	2	...	7
	♀̸	Under 35	Under 35	13	7	53	17–30	30–42	3	1	....	3	...	57
			Over 35	15	9	60	40–130	50–135	....	8	....	....	1	0
Under 35 . . .	♂	Over 35	Over 35	6	5	83	20–197	31–208	4	....	....	1	...	80
	♂̸	Over 35	Over 35	60	36	60	18–80	28–91	....	....	28	7	1	0
	♀	Over 35	Over 35	40	19	47	20–245	31–253	13	....	1	....	5	68
	Preg. ♀	Over 35	Over 35	16	9	50	20–203	31–211	7	1	....	....	1	87
	♀̸	Over 35	Over 35	64	29	45	20–68	31–79	....	....	22	4	3	0
Total . . .	......	....	....	327	173	53	....	....	....	....	....	....	...	...
Over 35	♂	Under 35	Under 35	39	7	18	13–21	89–109	2	....	....	4	1	28
			Over 35	4	0	0	24–28	65	....	....	....	....	...	0
	♂̸	Under 35	Under 35	30	5	17	13–21	94–109	....	3	....	2	...	0
			Over 35	5	2	40	24–35	125	....	....	....	1	1	0
	♀	Under 35	Under 35	42	3	7	13–20	89	....	....	....	2	1	0
	♀̸	Under 35	Under 35	14	3	21	20	100	....	....	....	3	...	0
			Over 35	5	0	0	20	100	....	....	....	....	...	0
Over 35	♂	Over 35	Over 35	23	8	35	20–203	66–251	7	....	....	1	...	87
	♂̸	Over 35	Over 35	15	1	6	20	84	....	....	....	1	...	0
	♀	Over 35	Over 35	39	3	8	20–70	84–118	....	1	....	2	...	0
	Preg. ♀	Over 35	Over 35	14	1	7	74	122	....	1	....	....	...	0
	♀̸	Over 35	Over 35	11	2	18	20	84	....	....	....	2	...	0
Total . . .	......	....	....	241	35	14	....	....	....	....	....	....	...	...
Grand Total	......	....	....	568	208	36	....	....	....	....	....	....	...	...

L.A. = light areas; Cast. = castrate; Undev. = undeveloped; Deg. = degenerate; No gl. = no gland tissue; %+ = percentage with light areas.

The colony of albino rats in our laboratory, although not closely inbred, gave, in general, fairly satisfactory results. Autotransplantations were done in some cases, and both littermate and nonlittermate combinations were made.

The tissue at transplantation was removed from a freshly killed donor and placed as rapidly as possible in subcutaneous sites on the abdomen of the host. At autopsy the transplants were found attached to the skin or body wall, from which they could be easily dissected for weighing. Usually 1 or 2 pieces of prostate and seminal vesicle weighing from 1 to 3 mg. were transplanted into each host. Castrations and spayings were done at the time the transplantations were made.

At autopsy of the hosts the grafts were removed, weighed, fixed in Bouin's fluid, and sectioned serially at 10 μ. In some grafts prostate cell heights and acinous diameters and seminal vesicle cell heights were measured for comparison with the host. The host reproductive tract was examined, and the ventral prostate and seminal vesicles of males were weighed, and samples were fixed for comparison with the grafts. In female hosts the ovaries were examined, and the uterine diameter measured. In spayed females an examination was made to determine that no ovarian regeneration had taken place, and the uterus was measured.

The weight of the recovered grafts varied from approximately 0.2 mg. to 123 mg. for the prostate, and from 0.4 mg. to 159 mg. for the seminal vesicle. It was not possible to predict the histological condition of such grafts from their weights. Within limits, however, one can say that very small grafts were more likely to contain only degenerate glands, fat, lymphocytes, and connective tissue; while very large grafts probably would contain more or less normal tissue, with light areas in the prostate and secretion granules in the seminal vesicles.

Table 1 presents a summary of the data on prostate grafts. The tissue was classified as under or over 35 days of age at transplantation, since 30–40 days had been shown to be a crucial age in the young, castrated rat prostate. However, since the main problem was to test out the ability of the young gland to differentiate, in the transplants listed as under 35 days of age tissue between 5 and 12 days of age was used in all but a few cases. A few transplants of tissue between 12 and 35 days of age were made. Transplants listed as over 35 days varied from 40 to about 150 days in age at transplantation.

The age of the host at the time of transplantation and autopsy was found to be the important factor in relation to the development and differentiation of prostate tissue in gonadless hosts; and in consequence the data were divided, according to the autopsy age of the host, into 35 days and under, and over 35 days.

Seminal vesicle grafts are summarized in Table 2 in the same form as the prostate grafts.

PROSTATE TRANSPLANTS

Since the interpretation of the condition of the grafts depended upon histological study, a brief review of prostate histology and the development of the gland is necessary. The ventral prostate of the rat consists of many acini which branch and coil. They lie close together (see Fig. 1) with a small amount of interacinous connective tissue and are lined by columnar epithelium in which the nuclei are basal. A distinct light band appears in the cytoplasm approximately halfway between the nucleus and the lumen end of each cell. At 5 days of age—the earliest stage at which transplants were made—the gland contains only solid cords of cells. These begin to hollow out by 10 days of age, and

TABLE 2

SUMMARY OF SEMINAL VESICLE TRANSPLANTS

DONOR	HOST			TRANSPLANTATIONS									
Age in Days at Transpl.	Sex	Age in Days		No. Made	No. Rec.	% Rec.	Histology						
		At Transpl.	At Autopsy				Sec. Grs.	Cast.	Simple	Compl.	Deg.	No Gl.	% +
Under 35......	♂	Under 35	Under 35	15	11	73	....	....	2	9	....	....	....
			Over 35	14	6	43	4	....	.....	2	....	....	67
	⚦	Under 35	Under 35	23	8	35	....	....	6	1	....	1	....
			Over 35	14	4	29	....	....	4	......	....	....	....
	♀	Under 35	Under 35	12	5	42	....	....	2	3	....	....	....
			Over 35	4	1	25	....	....	1	......	....	....	....
	♀̸	Under 35	Under 35	13	3	23	....	....	3	......	....	....	....
			Over 35	5	2	40	....	....	2	......	....	....	....
Under 35......	♂	Over 35	Over 35	7	7	100	6	....	.....	......	....	1	86
	⚦	Over 35	Over 35	14	10	71	....	....	6	3	....	1	....
	♀	Over 35	Over 35	39	18	46	....	....	8	7	2	1	....
	Preg. ♀	Over 35	Over 35	21	10	48	1	....	3	6	....	....	10
	♀̸	Over 35	Over 35	11	7	64	....	....	2	3	1	1	....
Total.....	....	....	....	192	92	48	....	....	.....	......	....	....	....
Over 35.......	♂	Under 35	Under 35	16	4	25	....	....	.....	......	3	1	....
			Over 35	3	0	....	....	....	.....	......	....	....	....
	⚦	Under 35	Under 35	6	1	17	....	....	.....	......	1	....	....
			Over 35	5	0	....	....	....	.....	......	....	....	....
	♀	Under 35	Under 35	10	2	20	....	....	.....	......	2	....	....
	♀̸	Under 35	Under 35	14	5	36	....	2	.....	2	....	1	....
			Over 35	5	0	....	....	....	.....	......	....	....	....
Over 35.......	♂	Over 35	Over 35	20	14	70	9	....	.....	......	5	....	64
	⚦	Over 35	Over 35	12	1	8	....	....	1	......	....	....	....
	♀	Over 35	Over 35	21	5	19	....	....	2	......	1	2	....
	Preg. ♀	Over 35	Over 35	9	3	33	....	3	.....	......	....	....	....
	♀̸	Over 35	Over 35	9	1	11	....	....	1	......	....	....	....
Total.....	....	....	....	130	36	27	....	....	.....	......	....	....	....
Grand total..	....	....	....	322	127	39	....	....	.....	......	....	....	....

Sec. Grs. = secretion granules; Compl. = complex structure; %+ = percentage with secretion granules.

and at 12 days many acini have lumina, and pale light areas are present (see Fig. 8). Further development involves enlargement of the acini, increases in epithelial height and strength of light areas, and growth of villous projections in the acini. By 25 days the gland is approximately like that of the adult, except that the gross size and the acinous size are smaller.

Castration of the adult involves, first, loss of cell light areas within 4 days and then progressive reduction in epithelial height, nuclear size, and acinous diameter. The epithelium becomes pseudostratified, and the nuclei pyknotic. Interacinous connective tissue appears to increase in amount as the acini grow smaller. As has been stated above, castration of the young rat within the first week of life does not result in regression of the ventral prostate.

It was possible by histological study of the grafts, using the criteria given above, to classify the tissue as undeveloped when the acini were small, round, and usually solid; normal when light areas were found in any of the acini; castrate; and degenerate. The condition of the acini in different parts of a graft frequently varied greatly. In some grafts the relation of the acini was normal (see Fig. 1), but in others less so (see Fig. 2), and in still others groups of acini were scattered in large amounts of connective tissue. Acinous size varied, and normal, quite distended acini containing epithelial light areas were often closely associated in the same graft with others that were small and castrate in appearance. Lymphocytes were usually present. Connective tissue was frequently abundant. In some grafts, particularly of older tissue, it completely obliterated the lumina of prostate acini and seminal vesicles. All these changes were observed in tissue from all classes of hosts and resulted from the abnormal conditions that necessarily obtain in tissue placed in a foreign environment and growing under the influence of foreign host reactions and changed vascularity. In spite of the somewhat abnormal appearance of many of the grafts, it was a relatively simple matter, in most cases, to determine the degree of development or regression.

A. TRANSPLANTS OF PROSTATE TISSUE UNDER 35 DAYS OF AGE AT TRANSPLANTATION

1. *Into young and old normal males.*—A few transplants of young prostates were made into normal males to test the ability of the tissue to survive and develop normally. A relatively high percentage of grafts was recovered (see Table 1), and, of these, 80–100 per cent possessed light areas in the epithelium. The histological character of the transplants varied from grafts that appeared normal to others that had only a few scattered acini and were in almost castrate condition. In some only a few acini showed light areas. It is true, however, that in normal prostate glands which are responding to a low male hormone level, as when castrates are injected with low dosages of male hormone, the acini enlarge from the castrate state; but frequently the peripheral acini are more nearly normal histologically than are other parts of the gland and have light areas when none can be found elsewhere. Thus there is, even in the intact gland, a differential response of the acini, seemingly associated with position in the gland.

Six transplants were made into older normal males, over 35 days of age at transplantation time; 5 were recovered, and 4 of these, or 80 per cent, had light areas. These grafts all showed great distention of the acini, greatly stretched and flattened epithelium, and large amounts of secretion. There was, of course, no outlet for secretion in the grafts; and the resulting accumulation of secretion and pressure made the epithleium, in some cases, flat, vacuolated, and abnormal. This factor of distention was found to be operating in grafts recovered from old normal males, some old normal females, and several pregnant females and appears to indicate a large amount of androgenic stimulation. The weights of the grafts, although not always dependable, do give additional support here to such a theory. The best positive grafts from older males weighed about 40 mg., as against 3.6 mg. for the best grafts from young males. Light areas were maintained in grafts for periods of a little over 6 months and could undoubtedly have been maintained longer.

2. *Into young and old castrated males.*—Approximately half of the transplants put into young castrated males survived, but several were degenerate or had no gland tissue. The grafts recovered from hosts over 35 days of age at autopsy differed from those recovered from younger hosts and will be treated separately.

Seven of the 17 grafts recovered from younger graft hosts had light areas. The best is illustrated in Figure 4. This tissue was transplanted from a 10-day-old male into a 5-day-old male (castrated when the transplant was made) and allowed to remain for 20 days, so that the host was 25 days old at autopsy and the graft tissue 30 days. The acini were distended, the epithelium moderately high, and light areas were somewhat pale but present consistently in many of the cells. A comparison of Figure 4 with Figure 3—the prostate of the host from which the graft was taken—shows that graft and host prostate were about equally well developed. Neither equals the development of the normal prostate of a 25- or 30-day-old normal male. The remaining 6 grafts were not so well differentiated, and, in some, very pale light areas were found in only a few cells. Two of the recovered grafts were definitely castrate in appearance, and 1 graft was classified as undeveloped and had small acini of a type that will be discussed later. It must be stressed that the prostates of young castrated males do not all show the same degree of differentiation; and they vary, at the age of 30 days, from an almost normal state to nearly castrate and in some few cases to a completely castrate one. The grafts show the same variability as the prostates of castrate males. Measurements of cell heights and acinous diameters showed that, in all but the examples shown in Figures 3 and 4, the acini of host prostates were more distended than those of the grafts and host epithelium was higher. The longest period of maintenance of cell light areas in castrated hosts was 20 days, when the host was 30 days old and the graft-tissue age was 34 days.

Young prostate transplants recovered from young castrates over 35 days of age when killed proved to be in a castrate state at that time, like the host prostate. Figure 5, the host prostate, and Figure 6, the graft, illustrate these findings. An autotransplant was made into an 11-day-old castrated host and left for 45 days. Host and graft were 56 days old when the host was killed. As Figures 5 and 6 show, both prostates were in typically castrate condition, with small acini and flat epithelium. Five of the 8 grafts in this category were like this, while 3 were degenerate or had no gland tissue. The number of grafts here is low, but grafts from the same-age spayed female and normal female hosts (usually under 80 days of age) gave the same results. One can conclude that grafts from castrated males killed below 35 days of age will have light areas and at least approach a normal functional state, while those from castrated hosts over 35 days of age at autopsy will show a castrate condition.

Some of the most interesting results were found in transplants of young prostate gland put into castrated males over 35 days of age at transplantation time. The grafts remained in the hosts for periods varying from 18 to 50 days. Twenty-eight of the 36 recovered grafts contained only small, undeveloped acini of the type shown in Figure 7. Ten-day-old prostate was put into a 47-day-old castrated male and allowed to remain for 18 days. The graft tissue in Figure 7 was 28 days old at autopsy, and the host 65 days old. The host prostate is not shown, but it was approximately like that in Figure 5 (a castrated male at 56 days) and was a typical castrate. The graft acini in Figure 7 can be seen to be relatively normal in appearance, with no indications of degeneration, and lymphocytes are not especially in evidence. The tissue resembles the normal young prostate at about 11 days of age (Fig. 8), except that some regression is evident as shown by the flat epithelium and small nuclei. A comparison of Figure 7 with Figure 6 shows the difference between tissue that has never developed to any marked degree and tissue that has developed as in Figure 4 and ultimately regressed. In some of the other 28 grafts the acini were smaller than those shown in Figure 7, and frequently all the acini were solid. Again, these 28 grafts varied, and some contained only a few acini, but these never were moderately or widely distended or had any indications of light areas. The graft in Figure 7 weighed 2.0 mg. as compared with 5.0 mg. for the graft shown in Figure 4 and 2.1 mg. for the graft in Figure 6. The 8 remaining grafts that did not show this type of acini were definitely degenerate.

3. *Into young and old normal females.*—Prostate transplants in young normal females killed at less than 35 days of age had light areas in 6 out of 9 recovered grafts. The remainder were degenerate. These 6 grafts varied as did all groups of grafts; but, in general, the range of acinous diameters and cell heights was about like that found in grafts from young normal males, and the grafts differed in no way histologically from grafts from males. The light areas seemed stronger and more consistent than those observed in grafts from young castrated males.

Transplants taken from females over 35 days of age at autopsy presented very different results, with the exception of 1 case. Ten of the grafts were typically castrate histologically, 1 had light areas, and 2 were degenerate. These findings were in accord with those found in castrated male hosts killed over 35 days of age. However, the female presents a more complicated situation. The 10 grafts that were typically castrate were recovered from hosts that were between 52 and 79 days old at autopsy, while the 1 containing distended acini, moderately high epithelium, and pale light areas was recovered from a 102-day-old female and had been transplanted for 90 days.

This difference in character of grafts from older females was still more marked in the grafts put into females over 35 days old at time of transplantation. Of 19 recovered grafts, 13 had light areas, 1 was undeveloped, and 5 had no gland tissue. Two of the positive grafts were taken from the same 71-day-old host, but all the other positive cases were from hosts ranging between 97 and 387 days of age at autopsy. The indications are that grafts from young females under 35 days of age will contain light areas in a high proportion of cases; from females between 35 and approximately 80 days of age the grafts will be mainly castrate, although an occasional female near the upper end of the age range may yield a positive graft (as indicated by 2 positive grafts from a 71-day-old female); from females over 80 or 90 days old the grafts will again contain light areas. The possible significance of this will be discussed later.

The graft in Figure 2 is typical of the 13 positive grafts obtained from older females. The tissue was transplanted at 15 days into a 141-day-old female host. After 42 days the graft was recovered, at 57 days of age, with the host 183 days old. The acini were somewhat scattered, but they appeared quite normal, and light areas were well marked. Grafts of young prostate tissue into older females were recovered approximately 8 months later and found to contain light areas. The largest graft in this group weighed 32.8 mg., which compares favorably with the largest from old normal males.

4. *Into pregnant females.*—Prostate transplants were also put into females that were already pregnant or were bred after transplantation of the tissue. When these females littered they were bred again, so that, for example, one female carrying a graft littered 7 times in 7 months. Nine grafts were taken from such females after periods ranging from 20 to 203 days. Seven of the grafts could be classified as appearing functional and possessing light areas, 1 was castrate, and 1 had only connective tissue. Several of the grafts had greatly distended acini and flat epithelium, as described above for grafts from old normal males, but others had more nearly the usual distention of acini, and the graft shown in Figure 1 is one of these. Eleven-day-old tissue was transplanted into a 68-day-old female that littered twice during the 68 days she was carrying the graft and was found to be pregnant again at autopsy. The host was 136 days old when killed, and the graft tissue was 79 days old. The graft weighed 52 mg. and was indistinguishable histologically from the prostate of a normal male except that there were no villi in these acini.

The grafts from pregnant females showed marked androgenic stimulation not only in the great acinous distention but also in the gross size of several of the grafts. The largest graft from old normal males weighed 39.8 mg. (unfortunately, not all grafts were weighed); from old females, 32.8 mg.; and the 3 largest from pregnant females, 33.4, 52.4, and 123 mg. The last is the largest prostate graft recovered in the course of the experiments. No estrogenic effects were observed in any prostate grafts recovered from normal young or old females or pregnant females.

5. *Into young and old spayed females.*—The transplants of tissue in these categories were found to duplicate the findings in young and old castrated male hosts. Young tissue put into young spayed female hosts killed under 35 days of age resulted in 7 grafts, 3 of which had light areas, 1 was castrate, and 3 were degenerate. In general, the best positive graft was similar to the graft in Figure 4 from a castrated male host. The longest maintenance of light areas was for a period of 30 days in tissue transplanted from a 10-day-old male to a 5-day-old spayed female. The host was 35 days old at autopsy, and the graft 40 days old. When the hosts were over 35 days at autopsy age, 8 castrate grafts were recovered, and 1 with no gland. The 8 grafts were typically castrate and resembled Figure 6.

Prostate grafts in older spayed females yielded the same result as in older castrated males. Twenty-two out of 29 grafts contained small acini like those shown in Figure 7.

B. TRANSPLANTS OF PROSTATE TISSUE OVER 35 DAYS OF AGE AT TRANSPLANTATION

It was found that prostate tissue from older rats was very limited in its ability to survive. Only 14 per cent of the transplants were recovered in comparison with 53 per cent when young tissue was used. The recovered grafts proved to be degenerate in 51 per cent of the cases, while only 15 per cent of the grafts of young tissue were degenerate.

The transplants of older prostate tissue were taken from males varying in age between 38 and 150 days. The prostates at the time of transplantation were, of course, well developed, and the problem was one of maintaining the differentiated condition.

When older prostate transplants were put into young hosts, a small number of grafts was recovered, as shown in the lower half of Table 1. It proved very difficult to obtain takes even in young males. Thirty-nine such transplants yielded only 7 grafts, and 2 of those had light areas, while 5 could be considered degenerate and lacking gland tissue. These 2 positive grafts were from the same host and were in approximately the same histological condition with only a few acini, and some of these were abnormal, some castrate, and a few had pale light areas. The host was 29 days old at autopsy, the tissue 89 days, and the transplantation period 20 days. From young castrated males killed under 35 days of age, only 5 grafts were recovered, and 3 were definitely castrate. In young castrated males killed over 35 days of age, in normal females, and in spayed females only a small percentage of grafts was found; and these recovered grafts were all so degenerate that no conclusions can be drawn as to whether old prostate tissue might be kept in a functional state in such hosts. The same holds true for all classes of old hosts except old normal males, in which 8 grafts were recovered from 27 transplants—a recovery of 35 per cent. Seven of these grafts were classified as functionally normal although some were nearly castrate. Others had moderately distended or widely distended acini with light areas. The graft weights in old normal males indicated stimulation, for they ranged from 4 mg. to 8 mg. Grafts in other hosts gave weights from 0.4 mg. to 3.4 mg., with 50 per cent of them weighing less than 1 mg.

SEMINAL VESICLE TRANSPLANTS

The seminal vesicles of the adult rat are paired structures containing a central lumen which runs the length of each gland and gives off ramified and complicated side branches. The epithelium is tall columnar with basal nuclei, and the cytoplasm contains prominent secretion granules surrounded by a clear area of cytoplasm or "halo."

The development of the seminal vesicle differs from that of the prostate in that it does not reach a differentiated state, as judged by the presence of secretion granules in the cells, until about 36 days of age, while the light areas of the prostate cells can be found at 11 or 12 days of age. At 5 days of age the histological structure of the seminal vesicle is very simple, for it consists of a central lumen from which small side branches are just beginning to grow. By 15 days the lateral growths are very complex, and at 30 days this is still more apparent, and the cells have become highly granular. The thirty-sixth day marks the beginning of true secretion granules in the cells, which by this age have increased to tall columnar. Further development includes great and rapid increases in gross size, cell height, and number and size of secretion granules.

Castration when the gland is in its adult, differentiated condition results in loss of the secretion granules within 48 hours and subsequent reduction in cell height, degenerative changes in the nuclei, and great loss in gross size.

If castration occurs within the first week of life, the seminal vesicles continue to develop to a limited degree. The side branches grow out from the central lumen, but the histological structure always remains simple, and no differentiation of the cells ever takes place. The gland grows slowly to about 30 days of age but does not usually exceed the size of that of a 15-day-old normal.

In the present experiments seminal vesicle tissue was transplanted from the same donors to the same respective hosts as were the prostate transplants in some of the earlier work. The results are given in Table 2. Although the number of grafts recovered is too small in many categories to give definite results, certain conclusions can be drawn as to the reactions of seminal vesicles when transplanted.

As with the prostate tissue, most of the young seminal vesicle transplants were between 5 and 12 days of age at the time of transplantation, with the great majority between 9 and 12 days of age.

It was difficult to classify clearly the seminal vesicle grafts, but the following categories were chosen: normal, with secretion granules, which meant that, in addition, the epithelium was high and the gland structure complex; castrate, when the graft showed the typical adult castration changes such as low epithelium and small nuclei; simple, when the lumen of the gland had few or no side branches; complex, when the side branches were ramified; degenerate; and with no glandular tissue. No further division of the simple and complex classifications was attempted on the basis of the state of the epithelium in such grafts. In some cases it was pseudostratified and with the cells crowded together; in others flat, squamous, and somewhat abnormal; and in still others definitely abnormal. No one type of epithelium was characteristic of tissue recovered from any one type of host. Host reactions were evident in the graft tissue in the form of lymphocytes and connective tissue infiltration, and these factors made it somewhat more difficult to interpret seminal vesicle reactions, particularly in regard to possible female hormone effects on fibrous and muscular tissue and epithelium.

A. TRANSPLANTS OF SEMINAL VESICLE TISSUE UNDER 35 DAYS OF AGE AT TRANSPLANTATION

1. *Into young and old normal males.*—Transplants of young seminal vesicles into young male hosts killed under 35 days of age (see Table 2) never had secretion granules, even though in several cases the graft tissue was over 40 days old. In grafts from young male hosts killed over 35 days of age, secretion granules were present in 4 grafts out of 6. The remaining 2, recovered from a host 38 days old, had a very complicated structure, but the grafts were evidently not receiving enough male hormone to elicit a completely positive response. In 2 out of the 4 positive grafts the graft tissue was only 33 days old, so the granules were formed somewhat precociously.

The weights of the grafts varied greatly, but the greatest androgenic stimulation, as judged by a weight of 125 mg., was found in a graft that had been transplanted for 155 days. The histological structure showed strong stimulation in the great distention of the lumen. Grafts that had been in normal young hosts for only 20–30 days usually weighed between 2 and 5 mg.

The seminal vesicle transplants in older males resulted in 6 out of 7 grafts with complicated structure and secretion granules. Distention in the lumina of the grafts frequently caused a stretching of the epithelium so that it was low and sometimes abnormal, but this seemed, as in the prostate grafts, the result of accumulation of secretion. In older male hosts there was evidence of far greater amounts of male hormone available for the grafts than in the young males, for positive grafts that had been in the host only 20 days weighed from 17 to 35 mg. and one with a graft period of 197 days weighed 159 mg.

2. *Into all other hosts, young and old.*—In the remainder of the grafts recovered from young hosts of all other classes, the histological structure of the grafts varied from a simple, open lumen to a moderately complex one, but no stimulation of the epithelium was ever found. This is in direct contrast to the findings in prostate grafts. No estrogenic effects on epithelium, fibrous tissue, or muscle were observed in the 6 grafts recovered from normal females, with 1 possible but questionable exception in which there seemed some hypertrophy of muscle.

In grafts from older hosts the tissue again varied from simple to complex, but evidence of androgenic stimulation was found in 2 grafts recovered from a female that had carried the grafts for 147 days and littered 6 times in that period. One of the grafts had very complicated structure, fairly high epithelium, and numerous granules, some with halos. The lumina were filled with secretion. The second graft was also very complicated and had a large amount of secretion in the lumen, but the epithelium was not so high and the granules not distinct. Both grafts gave the appearance of gland tissue that had been secreting. The graft weights were 28 mg. and 17 mg. A ventral prostate graft from the same host had distended acini and some high epithelium but no light areas and seemed in an early stage of regression.

It was difficult to recognize definite estrogenic effects because, as has been discussed, increases in connective tissue and abnormal epithelium were factors met, to some degree, in all classes of hosts, although they did not seem so evident in grafts from normal males. In some of the grafts from old females and from those bred repeatedly there were possible indications of muscle hypertrophy, but it was never striking. The weights of the recovered grafts, however, suggest that there may have been estrogenic influence, for the grafts from old females weighed, in over one-third of the cases, between 10 and 25 mg., while those from spayed females and castrated males weighed between 1 and 6 mg. This was definitely not a functional stimulation such as was found in grafts from male hosts, with the exception of the 2 cases noted above from a pregnant female. The increased weights of the grafts from females seemed to be caused by increase in connective tissue and possibly by muscle hypertrophy.

B. TRANSPLANTS OF SEMINAL VESICLE TISSUE OVER 35 DAYS OF AGE AT TRANSPLANTATION

In the experiments in which older seminal vesicle tissue was transplanted (see lower half of Table 2), only 27 per cent of the grafts were recovered, and almost one-half of these were degenerate or had no gland tissue. The numbers are so low that it is impossible to draw any conclusions except that adult seminal vesicle tissue was maintained in a functional state with large secretion granules in old male hosts for as long as 7 months. Again, the grafts varied in weight, but functional grafts weighing 38 mg. were recovered. No graft from any other host weighed more than 4 mg.

DISCUSSION

The transplantation of young ventral prostate tissue into young and old castrated males and spayed females was designed to bring out, if possible, whether such tissue has the capacity to differentiate regardless of the age or sex of the host. The results show that young prostate tissue transplanted into young castrated males and spayed females develops distended acini and epithelial light areas in a certain percentage of cases. This differentiation in the grafts resembles and parallels that of the prostate gland of the young castrated male rat and is maintained until the host is about 35 days of age, when regression begins.

When young prostate transplants are put into hosts which are over 35 days old at the time of transplantation, the tissue survives and grows in some cases, but none of the grafts ever develop acini that are widely distended or have any evidence of epithelial light areas. These findings lead to the conclusion that young prostate tissue does not seem to be capable of "self-differentiation" but is dependent upon androgenic stimulation, which is provided in the young castrated male and spayed female rat and not in older castrated males and spayed females. The source of such androgenic stimulation is the next question.

Howard (1937, 1938) corroborated the findings of differentiation of the ventral prostate in young castrated rats as reported by Price (1936) and suggested that the source of andromimetic material that could be capable of stimulating the prostate might be the juvenile cortex of the rat adrenal, since it is present at the time the prostate of the young castrated rat is developing to a differentiated state and has lost its distinctive character at about 35–40 days of age, when the prostate regresses. In further work Howard (1939) has shown a close relationship between the histological condition of mouse seminal vesicles and prostate and the presence in the adrenal cortex of either a primary or a secondary X zone. In rats Howard found that castration did not result in hypertrophy of the X zone, nor did the seminal vesicles differentiate after castration at ages corresponding to those at which castration was done on mice.

Other research gives further support to the theory that the adrenal gland may be responsible for androgenic stimulation of the prostate. Davidson and Moon (1936) administered adrenocorticotropic hormone to young castrated male rats and caused hypertrophy of the adrenal cortex and gross and histological stimulation of the prostate. Burrill and Greene (1939*a*) castrated a group of young male rats, castrated and adrenalectomized others, and adrenalectomized a third group. The prostates of the castrated rats and of the adrenalectomized rats were found at autopsy to be differentiated and contained epithelial light areas, but those of the rats that were both castrated and adrenalectomized were castrate in histological appearance. Gersh and Grollman (1939) performed somewhat similar experiments, with conflicting results. They used rats and mice that were castrated or castrated-adrenalectomized. All the latter animals were maintained by the oral administration of a cortical hormone which had been tested on castrated mice and had shown no androgenic activity. The size of tubules and cells in two lobes of the prostate and in Cowper's gland was measured, and the relative volume of the glands was computed. They found no difference between the glands of castrated and castrated-adrenalectomized animals. Their conclusion was that the adrenal cortex, more particularly the "juvenile" cells of rats and the X zone of mice, has no androgenic function.

Still other lines of evidence point to the adrenal cortex as a source of androgenic substance. Reichstein (1936, 1938), Hodler (1936, 1937), and Parkes (1937) found such a substance in adrenal extracts. Gersh and Grollman (1939) failed to demonstrate any such activity in extracts of adrenals of fetal pigs, fetal and newborn humans, and in extracts of X-zone-bearing adrenals of mice. In various clinical pathologies with adrenal involvement large amounts of androgenic substance have been recovered from the urine.

Although there is some disagreement as to the importance of the adrenal as a source of androgens, the evidence suggests that under certain conditions the adrenal cortex is capable of elaborating such substances in effective amounts and that the prostate of the rat and the prostate and seminal vesicles of the mouse respond, as evidenced by their behavior in the castrated animal. The seminal vesicles of the rat have been shown to have a much higher male hormone threshold than the rat ventral prostate, and this may account for their failure to differentiate in the presence of possible adrenal androgens.

The question of the amount of adrenal androgens circulating normally in rats is an interesting one. Regression of the prostate and seminal vesicles of adult rats follows within a few days after castration. This indicates (1) that the adult prostate tissue is less reactive or (2) that less extra-gonadal androgen is present in adults or (3) that both conditions obtain. It is possible that adult prostate requires more male hormone to maintain its normal histological state than young prostate tissue. However, the results of transplanting young prostates into old castrated males and spayed females show that there is definitely a smaller amount of extra-gonadal androgen in adult castrates and spays, for the transplants fail to differentiate although the tissue incorporates and grows. Howard (1939) has shown that castration in the adult rat does not result in differentiation of the adrenal cortex.

It must be assumed that in the young rat, both male and female, there are normally present androgens of adrenal origin, unless it is possible that removal of the gonads brings into action or increases a potential and temporary capacity. There are several facts that tend to disprove this latter suggestion and to indicate that some young normal females have adrenal androgens in effective amounts. Transplants of male ventral prostate tissue into young normal females have been shown in this work to differentiate and develop light areas, as do transplants in spayed females; and in both types of host the transplants are castrate in appearance when the hosts are over 35 days old at autopsy. In normal female hosts this is only a temporary condition, and older female hosts again yield positive grafts. A study of the normal development of spontaneously occurring female prostates (Price, 1939) brings out that in normal females in which such glands occur the female prostate develops light areas resembling those of the male prostate and maintains them until about 40 days of age, when the gland becomes castrate. The female prostates are highly variable in the degree of development they attain, and in this respect and in time of regression resemble the male prostate of the young castrated male. It was suggested (Price, 1939) that the development and regression of the prostate of normal females might depend upon the adrenal cortex. In work to be published elsewhere, it has been shown that female prostates can develop and differentiate in the absence of the ovaries. This has been reported recently by Burrill and Greene (1939*b*).

There is, however, a possibility that young ovaries produce small amounts of androgenic substance that may reinforce the action of adrenal androgens, so that the development of the female prostate in the young female may normally be influenced by both. The present findings in prostate transplants from young normal females and young spayed females give some support to this. The number of recovered grafts is too low to be more than suggestive; but, although grafts containing light areas were recovered from both types of hosts when autopsy was done under 35 days of age, there were indications in epithelial height, acinous diameters, and light areas that grafts from normal female hosts showed stronger stimulation. Further experiments are now in progress to test this point.

Burrill and Greene (1939*b*) report that transplants of male prostate put intraperitoneally in young normal females and spayed females that were killed at 20–35 days of age were always nonfunctional. Female prostates were found in 4 of the intact implanted females and showed evidence of functional activity. The spayed female hosts gave nonfunctional male grafts; but, out of 10 spayed females with female prostates, 4 of the prostates showed some evidence of function. From this they concluded that the threshold of reaction of female prostate tissue is below that of male prostate. In further experiments (Greene and Burrill, 1939*b*) they implanted young normal and spayed females with male ventral prostate tissue and injected the hosts with chorionic gonadotropic hormone. Positive implants were recovered from the normal female hosts but not from the spayed hosts, and the clitora of the normal females were enlarged. In hosts older than 28 days the implants were negative. Their conclusions were that the young, immature ovary can be stimulated to produce androgens in amounts sufficient to induce a functional state in male prostrate grafts but that in older females the ovary no longer can be stimulated.

The findings of Burrill and Greene (1939*b*) that male prostate grafts were nonfunctional in young normal and spayed female hosts between 20 and 35 days of age are not in agreement with the results reported here. It must be stressed that prostate tissue put into a foreign position in a host is not entirely comparable to the host prostate. Implants are subjected to a number of unfavorable influences, one of which is changed vascularity. In our work it was found that in almost all cases grafts showed a lower degree of androgenic stimulation as judged by a comparison of epithelial height and light areas and acinous diameters than the host prostate. In our experiments in male prostate transplants recovered from young normal females and spayed females there was variability in histological condition, but there was no question as to the functional state of some of the grafts.

Grafts recovered from female hosts older than 35 days but younger than 80 days were castrate in histological condition. This could be interpreted to mean, since that age range corresponds to a time when the adrenal androgens would be expected to disappear or be markedly reduced in amount, that the grafts reflected the lack of adrenal stimulation. The findings of Bradbury and Gaensbauer (1939) and Greene and Burrill (1939*b*) tend to suggest that the young ovary is limited in the period in which it can be stimulated to produce detectable amounts of androgen. The present transplantation experiments indicate that young ovaries may normally be producing a small amount of androgenic substance. If the ovary is limited in its androgenic capacity, the lack of functional activity in grafts in females in the period from approximately 35 days to nearly 80 days may possibly be due to a drop in androgens, both from the adrenals and from the ovaries.

Although the young ovary may or may not be normally producing effective amounts of androgens, there is no question that ovaries of adult rats do so. Male prostate grafts in old females (over 80 days of age) were developed and maintained in normal histological condition for months. This was true both for virgin female hosts and for those bred continuously. No differentiation was ever attained by grafts in old spayed females. It was suggested (Price, 1939) that the normal female prostate might have a higher hormonal threshold than the male prostate from the following facts. Male prostate grafts in old females are functional, as reported by Price (1937) and in the present paper. All normal female prostates found so far in old females have been in a somewhat castrate state, although not greatly regressed.[2] Price (unpublished) has found that male prostate grafts recovered from old females having normal female prostates show light areas, while the female prostate, in spite of its more favorable position, has a histological state below that of the graft. Burrill and Greene (1939*b*) have interpreted their results to mean that the female prostate has a lower hormonal threshold, because in their work male prostate grafts were always nonfunctional in young females, even though the host female prostate was functional. A series of experiments on threshold differences as studied by male and female prostate transplants is in progress and will be reported elsewhere.

Other workers have shown that ovaries can stimulate male accessory glands. Hill and Gardner (1936), Hill (1937*a* and *b*), Hill and Strong (1938), and Deanesly (1938) have shown that ovarian grafts put into the ears of castrated male mice and rats will maintain the reproductive accessories, prostate, and seminal vesicles at a normal size and in a normal histological condition. Hill attributes the androgenic activity to the lowered temperature of the ear, which he assumes brings out a potential capacity of the female gonad. Deanesly correlates androgenic activity of the ovary with thecal luteinization of the ovarian graft.

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[2] While this paper was in press, functional female prostate glands with cell light areas present were found in two normal adult female rats.

Ovaries have been shown by many workers to produce substances with androgenic action under a variety of conditions. Transplanted ovaries have maintained normal male accessory glands in castrated guinea pigs (Lipschütz, 1932) and mice (De Jongh and Korteweg, 1935). Removal of one ovary and partial removal of the other caused masculinization of female guinea pigs (Lipschütz, 1933, 1937). Steinach and Kun (1931) produced masculinization of female guinea pigs by irradiation with x-ray and with injection of gonadotropic substances. Guyénot and his collaborators have obtained masculinization of female guinea pigs by injection with extracts of hypophyses and with urine of pregnant women (Guyénot, Ponse, and Wietrzykowski, 1932; Guyénot, Ponse, Dottrens, Vallette, and Trolliet, 1933; Guyénot and Naville-Trolliet, 1936) and observed masculinization in female guinea pigs of an interspecific cross—*Cavia aperea* ×*C. cobaya* (Guyénot and Duszynska-Wietrzykowski, 1935). Papanicolaou and Falk (1934) also reported masculinization of female guinea pigs by injection of pregnancy urine extracts. Recently Greene and Burrill (1939*a* and *b*) and Bradbury and Gaensbauer (1939) have induced masculinization in young female rats with gonadotropic extracts.

All these researches, including the present report show that ovaries of some female mammals are capable of producing substances which stimulate male accessory gland and have an androgenic action on the clitoris of females. Luteinization or thecal luteinization of the ovaries has been thought by some workers to be associated with such androgenic action. It has been shown that extracts of corpora lutea, progesterone, and progestin are capable of stimulating male accessory glands in young castrated rats (Lamar, 1937; Greene, Burrill, and Ivy, 1939). The latter suggest that the androgenic action produced by transplanted ovaries and by those stimulated with A.P.L. may be due to progesterone. Hill and Strong (1938) and Deanesly (1938) conclude that transplanted ovaries produce an androgen that is not testosterone. The nature of the ovarian-produced androgen seems to be still an open question.

No estrogenic effects were observed in prostate grafts put into normal females. In seminal vesicle grafts there were indications in the histology and also in graft weights that host ovaries might be causing some increase in fibrous and muscular tissue. Pfeiffer (1936) transplanted ovaries into castrated male rats and reported hypertrophy of muscle in host seminal vesicles and coagulating glands. The epithelium of the coagulating gland became stratified, squamous, and, in extreme cases, cornified. Hyperplasia and metaplasia were found in the lateral lobe of the prostate.

Transplants of prostates and seminal vesicles have been used by several workers as indicators of male hormone presence or as a means of studying the reactions of these glands in the presence of introduced hormones. Fisher (1923) transplanted guinea pig and rat seminal vesicles and showed that seminal vesicle grafts would persist and appear functional in normal male hosts. Heckel and Kretschmer (1935) made autotransplants of rabbit prostate into the anterior chamber of the eye and obtained successful growth, which could be observed easily. Some increase in size of the transplants was noted after injection of antuitrin S, and hyperplasia and hypertrophy of the glandular structure occurred. R. A. Moore and collaborators (Moore, Rosenblum, Tolins, and Melchionna, 1937; Moore, Melchionna, Tolins, and Rosenblum, 1937; Moore and Smith, 1937) transplanted rabbit prostate and seminal vesicle into the anterior chamber of the eye and photographed the implants daily. They found a daily variation in size, which was markedly diminished or absent after castration. Slight or moderate temporary increase in size of the transplant in normal males was observed after injections of gonadotropic substance from pregnancy urine, alkaline extracts of anterior pituitary, male hormone, and female hormone. In castrated males all pituitary derivatives were ineffective, but male and female hormones caused increase in the size of the grafts. Corpus luteum hormone had no effect. A correlated elevation of blood cholesterol and increase in size of ocular transplants were found in male rabbits injected with female sex hormone. Ingestion of cholesterol had no effect on transplant size. In all this work no differences in reaction were found between the prostate and the seminal vesicle.

Melchionna and Flanders (1938) transplanted seminal vesicle tissue into the eyes of female rabbits and found that daily variations in size occurred in female hosts and were less conspicuous when the hosts were spayed. Injections of gonadotropic hormone caused slight increases in size, and both male and female hormones produced size increases in implants in normal and spayed hosts. Corpus luteum injections had no effect.

Turner (1938) used intraocular transplants of rat prostate and seminal vesicle as indicators of hormone production of intraocular testis transplants.

SUMMARY AND CONCLUSIONS

I. Experimental results
  A. Young ventral prostate transplants will grow and differentiate and possess a normal functional histological state:
    1. In young or old normal males for long periods.
    2. In young normal females until a host age of approximately 50–60 days.
    3. In young normal females killed at a host age of over 80 days or in old normal females (virgin or bred and lactating) for months.
    4. In young castrated males, or spayed females, to a host age of about 40 days.
  B. Young ventral prostate transplants will be incorporated and be maintained but will undergo little or no development beyond their stage at time of transplantation:
    1. In old castrated males.
    2. In old spayed females.
  C. Old ventral prostate tissue will be maintained in a normal functional histological condition:
    1. In old normal males for months.
    2. In young normal males for months.
  D. Young seminal vesicle transplants will grow and differentiate and possess a normal functional histological state:
    1. In young or old normal males for months.
    2. Possibly in some females that have been bred repeatedly for long periods.
  E. Old seminal vesicle transplants will grow and differentiate and possess a normal functional histological state:
    1. In old normal males for months.

II. Conclusions
  A. Ventral prostate tissue, which is normally under control of male hormone from testes, is stimulated by androgens from the adrenals in young castrated males,

young normal females, and young spayed females, and by ovarian androgens in older females, and possibly to some degree in young females.

B. Seminal vesicle tissue presumably has too high a hormone threshold to react to these influences except for an indication of androgenic stimulation in grafts in old females bred repeatedly, in which case ovarian androgens were probably effective.

LITERATURE CITED

BRADBURY, J. T., and GAENSBAUER, F. 1939. Proc. Soc. Exper. Biol. and Med., **41**:128.
BURRILL, M. W., and GREENE, R. R. 1939*a*. Proc. Soc. Exper. Biol. and Med., **40**:327.
——. 1939*b*. *Ibid.*, **42**:764.
DAVIDSON, C. S., and MOON, H. D. 1936. Proc. Soc. Exper. Biol. and Med., **35**:281.
DEANESLY, R. 1938. Proc. Roy. Soc. London, B, **126**:122.
FISHER, N. F. 1923. Amer. Jour. Physiol., **64**:244.
GERSH, I., and GROLLMAN, A. 1939. Amer. Jour. Physiol., **126**:368.
GREENE, R. R., and BURRILL, M. W. 1939*a*. Proc. Soc. Exper. Biol. and Med., **40**:514.
——. 1939*b*. *Ibid.*, **42**:761.
GREENE, R. R.; BURRILL, M. W.; and IVY, A. C. 1939. Endocrinology, **24**:351.
GUYÉNOT, E., and DUSZYNSKA-WIETRZYKOWSKA, MME. 1935. Rev. suisse zool., **42**:341.
GUYÉNOT, E., and NAVILLE-TROLLIET, MME I. 1936. Rev. suisse zool., **43**:415.
GUYÉNOT, E.; PONSE, K.; DOTTRENS, E.; VALLETTE, M.; and TROLLIET, I. 1933. Rev. suisse zool., **40**:217.
GUYÉNOT, E.; PONSE, K.; and WIETRZYKOWSKA, J. 1932. Compt. rend. Acad. sci., **194**:1051.
HECKEL, N. J., and KRETSCHMER, H. 1935. Surg., Gyn. and Obst., **61**:1.
HILL, R. T. 1937*a*. Endocrinology, **21**:495.
——. 1937*b*. *Ibid.*, p. 633.
HILL, R. T., and GARDNER, W. U. 1936. Anat. Rec., Suppl., **64**:21.
HILL, R. T., and STRONG, M. T. 1938. Endocrinology, **22**:663.
HODLER, D. 1936. Compt. rend. Soc. de biol., **122**:512.
——. 1937. Arch. d'anat., d'histol., et d'embryol., **24**:1.
HOWARD, E. 1937. Amer. Jour. Physiol., **119**:339.
——. 1938. Amer. Jour. Anat., **62**:351.
——. 1939. *Ibid.*, **65**:105.
JONGH, S. E. DE, and KORTEWEG, R. 1935. Acta brev. neerl., **5**:126.
LAMAR, J. K. 1937. Anat. Rec., Suppl., **70**:45.
LIPSCHÜTZ, A. 1932. Virchow's Arch. f. Path. Anat., **285**:35.
——. 1933. Compt. rend. Soc. de biol., **112**:1272.
——. 1937. Nature, **140**:892.
MELCHIONNA, R. H., and FLANDERS, S. 1938. Endocrinology, **23**:468.
MOORE, R. A.; MELCHIONNA, R. H.; TOLINS, S. H.; and ROSENBLUM, H. B. 1937. Jour. Exper. Med., **66**:281.
MOORE, R. A.; ROSENBLUM, H. B.; TOLINS, S. H.; and MELCHIONNA, R. H. 1937. Jour. Exper. Med., **66**:273.
MOORE, R. A., and SMITH, J. J. 1937. Jour. Exper. Med. **66**:291.
PAPANICOLAOU, G., and FALK, E. A. 1934. Proc. Soc. Exper. Biol. and Med., **31**:750.
PARKES, A. S. 1937. Nature, **139**:964.
PFEIFFER, C. A. 1936. Anat. Rec., **65**:215.
PRICE, D. 1936. Amer. Jour. Anat., **60**:79.
——. 1937. Anat. Rec., Suppl., **70**:60.
——. 1939. Proc. Soc. Exper. Biol. and Med., **41**:580.
REICHSTEIN, T. 1936. Helvet. chim. acta, **19**:223.
——. 1938. Ergebn. d. Vitamin- Hormon-Forsch., **1**:334.
STEINACH, E., and KUN, H. 1931. Arch. f. d. ges. Physiol., **225**:266.
TURNER, C. D. 1938. Amer. Jour. Anat., **63**:101.
WIESNER, B. P. 1934. Jour. Obst. and Gyn. Brit. Emp., **41**:867 and **42**:8.

PLATE I

FIG. 1.—Ventral prostate graft 79 days of age. Transplanted at 11 days of age into a 68-day-old female and allowed to remain 68 days. Host 126 days old at autopsy and had littered twice during the graft period and was pregnant again at autopsy. Note the distention of the acini and their normal relationship. Light areas in the cells are apparent. ×250. Graft weight, 52 mg.

FIG. 2.—Ventral prostate graft 57 days of age. Transplanted at 15 days of age into a 141-day-old virgin female and allowed to remain for 42 days. Host 183 days old at autopsy and had remained unbred. Note the somewhat abnormal relationship of the acini and their small size, but the presence of high epithelium and strong cell light areas. ×250. Graft weight, 3 mg.

FIG. 3.—Ventral prostate of 25-day-old castrated male, host for graft in Figure 4. Castrated at 5 days of age. ×250.

FIG. 4.—Ventral prostate graft 30 days of age. Transplanted at 10 days into a 5-day-old castrated male and allowed to remain for 20 days. Host 25 days old at autopsy. Note similar acinous distention and cell light areas in graft and in host prostate in Figure 3. ×250. Graft weight, 5 mg.

PLATE II

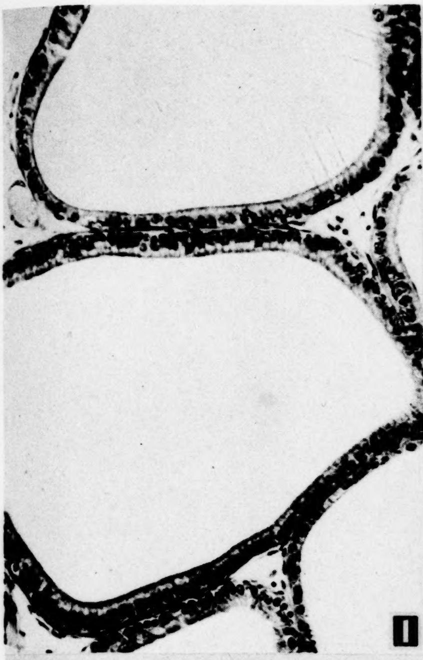
FIG. 5.—Ventral prostate of 56-day-old castrated male, host for graft in Figure 6. Castrated at 11 days of age. ×250.

FIG. 6.—Ventral prostate graft (autotransplant) 56 days of age. Transplanted at 11 days and allowed to remain for 45 days. Host 56 days old at autopsy. Note castrate condition of host prostate (Fig. 5) and prostate graft. ×250. Graft weight, 2.1 mg.

FIG. 7.—Ventral prostate graft 28 days of age. Transplanted at 10 days into a 47-day-old male (castrated at the time of implantation) and allowed to remain for 18 days. Host 65 days old at autopsy. Note the small acini and small dark nuclei and compare with Figure 8. ×250. Graft weight, 2 mg.

FIG. 8.—Ventral prostate 11 days of age. Tissue typical of young ventral prostates at the time of transplantation. Note the presence of some high epithelium and pale light areas. ×250.

PLATE I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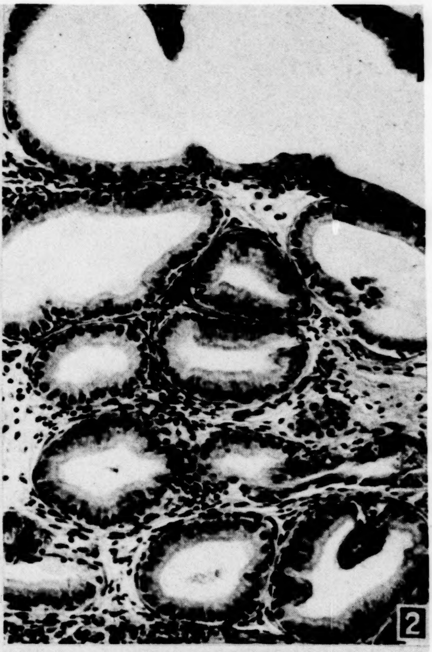
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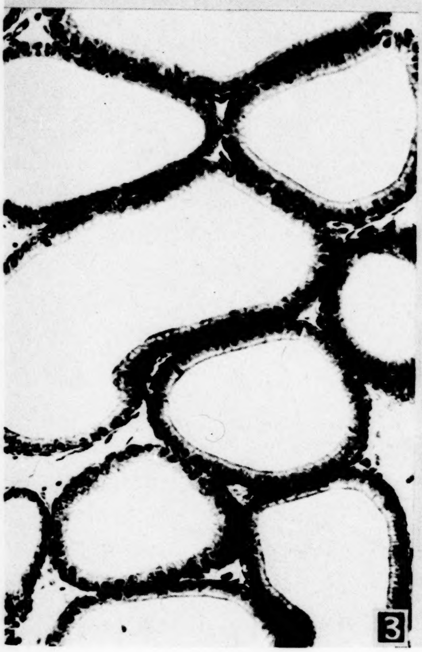
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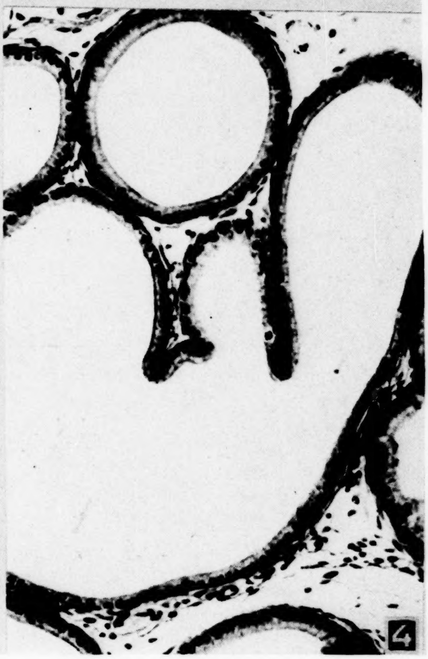
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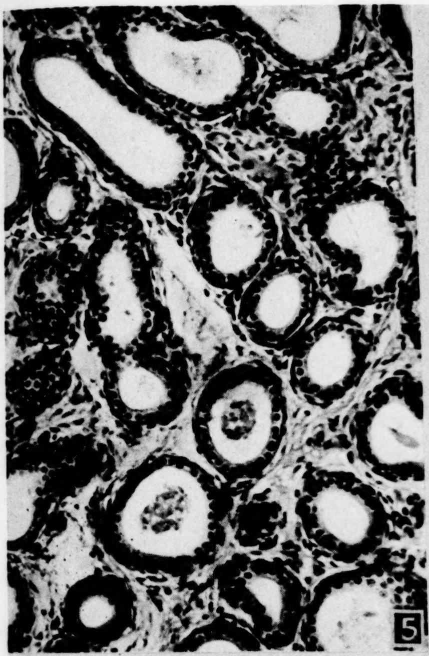
PLATE II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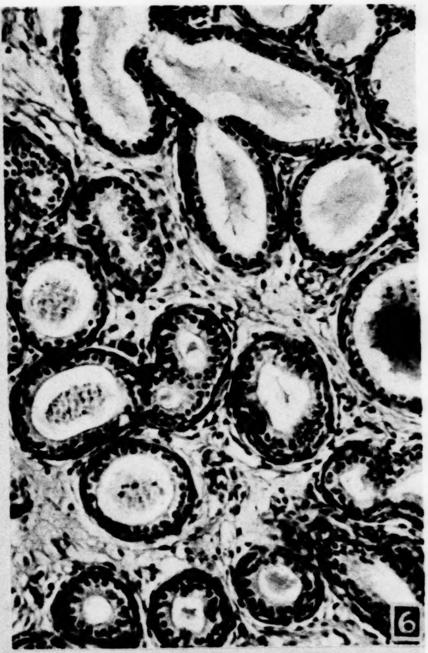
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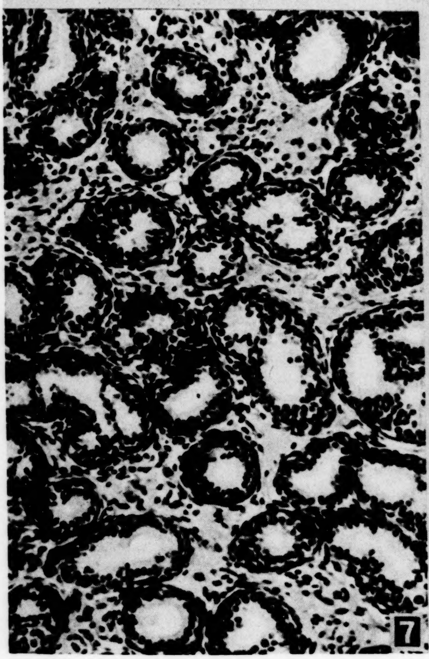
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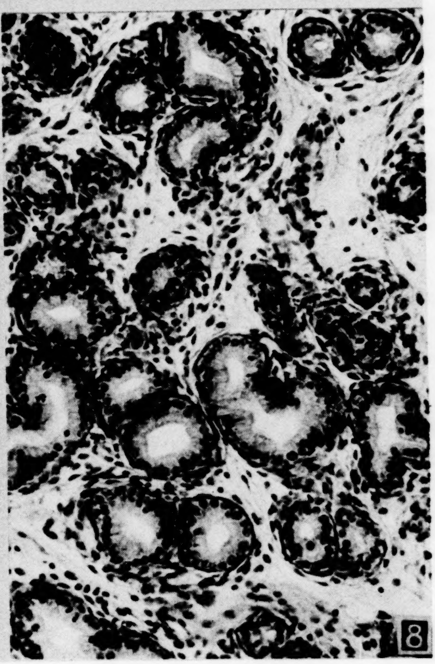
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SEQUENCE OF ROENTGEN RAY EFFECTS ON THE DEVELOPMENT OF THE GRASSHOPPER EGG. I. EFFECTS ON SUBSEQUENT DEVELOPMENT OF IRRADIATING 1-DAY EGGS[1]

(Seven figures)

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MOST of the investigations on biological effects of roentgen radiation have been restricted to a single criterion. In many cases the effect studied has been cytological injury, in some it has been retardation of growth, and in others the percentage of individuals killed. Few have attempted the correlation of these cytological and physiological effects. Such a correlation is of interest in determining which changes after irradiation are primary and which are secondary in nature. The grasshopper (*Melanoplus differentialis*) egg is a favorable material for such a study because it is well known both from the morphological and from the physiological standpoints.

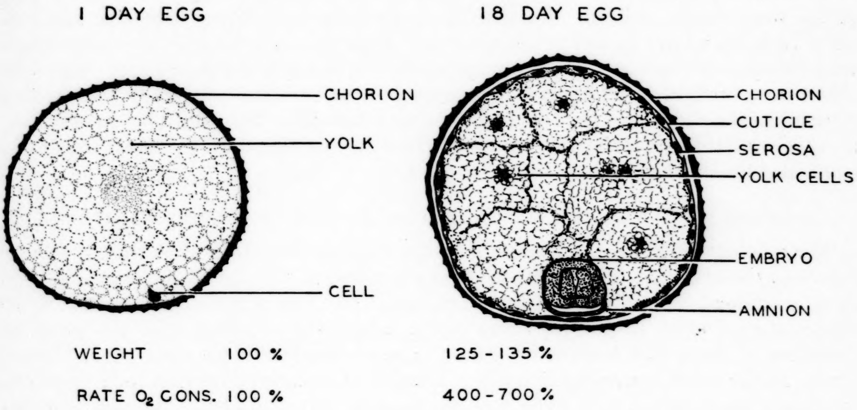

FIG. 1.—Diagrammatic cross-sections to show some of the changes which take place in 18 days at 25° C.

Several changes occur during the development of the grasshopper egg which respond to x-radiation, and these may be used as measurements of radiation injury. Figure 1 shows, diagrammatically, some changes which occur during the first 18 days of development at 25° C. These changes which can be studied quantitatively, are (1) growth of the embryo, (2) production of yolk and serosa cells, (3) increase in rate of oxygen consumption, and (4) increase in gross weight. It is possible, by using these as criteria, to employ several methods of studying radiation injury on the same organism. The relative radiosensitivity of the various processes undergoes marked change at several stages in the development of the egg (Evans, 1934, 1935, 1936*a* and *b*, 1937, 1938). For this reason the investigation has been divided into several parts, each dealing with a particular stage in development. This report is the first of the series and deals with effects (on subsequent development) of irradiating the eggs on the first day after laying. The effects of radiation on 1-day eggs are in marked contrast to those which occur in eggs rayed at other stages of development.

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[2] It is a pleasure to acknowledge the assistance of Professors J. H. Bodine and H. D. Kerr, whose interest and help have made this investigation possible. Thanks are due also to Dr. E. H. Slifer for her constructive criticism and for the loan of the slides of whole eggs, which were used in preparing the diagrams of Fig. 1.

MATERIAL AND METHODS

The radiation (130 kv., 5 ma., cardboard filter only, target distance, 30 cm.) was applied in one treatment, and the amount was increased by lengthening the exposure time.

The sand in the grasshopper cages was cleared of eggs at 10:00 P.M., and the eggs were collected at 8:00 A.M. the next day. Unless otherwise specified, the eggs used in this investigation were irradiated in the afternoon of the first day so that they ranged in ages from 12 to 24 hours when exposed to the radiation. Eggs from several pods were used so that each lot consisted of from 100 to 300 eggs at the beginning of the experiment. Eggs were taken from each lot at intervals of 2–3 days, and the embryos examined. The reader is referred to the paper of Slifer (1932) for the morphological changes during development at 25° C. All these experiments were performed at this temperature, and moisture was controlled by keeping the eggs on moist sand.

EFFECTS OF THE RADIATION ON DEVELOPMENT AND RATE OF OXYGEN CONSUMPTION

The most striking result of irradiating 1-day eggs was the all-or-none character of the injury to the embryos. The irradiated eggs examined at the end of the experiment (30 days) contained either a whole embryo or none at all. This is in marked contrast to the results of irradiating older eggs, where partial injury was obtained. This change in the character of the injury has been reported in more detail in a previous paper (Evans, 1937). In the present investigation it was found that dosages of 100 and 200 r prevented the formation of embryos in some eggs but that the development of the surviving embryos was not visibly affected except that they appeared to be growing more slowly than the controls. The effect of retarding the development of the embryo was more evident following dosages of 400 and 500 r.

The rate of morphological development was closely paralleled by the increase in oxygen-consumption rate. The measurements of oxygen consumption were made at 25° C. in small Krogh respirometers of the type developed in this laboratory. Determinations were made on lots of 20 eggs, and the same manometer was used for each experimental lot and its control throughout the experiment. The curve of oxygen consumption for the controls during the experiment is shown in Figure 2 (Curve 1). It will be observed that the rate of oxygen consumption increases very rapidly after the fourth day and reaches a maximum about the eighteenth. The rate then decreases to a minimal level and remains low for an indeterminant period (diapause).

The retardation of developmental rate and oxygen consumption, as a result of irradiation with moderate dosages, is shown in Curves 2 and 3. The lower peak of the experimental curves is probably related to the fact that many of the irradiated eggs contained no embryos. It will also be observed that the oxygen-consumption rates (Curves 2 and 3) did not drop to the level of the controls at diapause by the twenty-seventh day. This was probably due to the fact that the embryos had not reached the morphological stage at which the controls go into diapause and were, therefore, still developmentally active. Later the embryos ceased growing, and the oxygen-consumption rate dropped to the minimal level. This phenomenon of diapause has been studied extensively by Bodine (1923, 1929, 1932*a*, and 1932*b*).

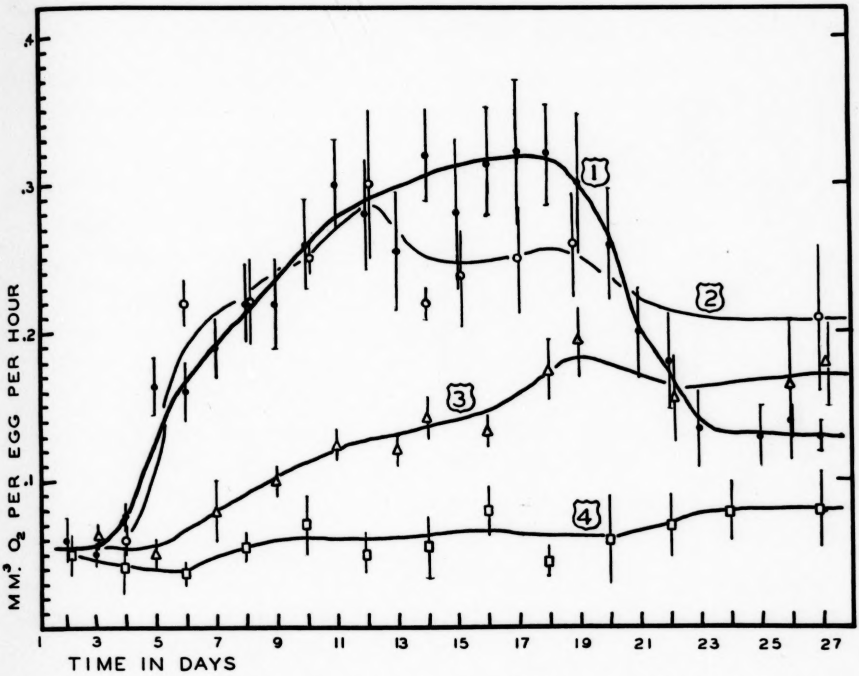

FIG. 2.—Effects of roentgen radiation on oxygen consumption and development. The means and standard errors of the means of 17 lots of controls shown in Curve 1. Curve 2 is of means and standard errors of 4 lots of eggs rayed 100–300 r. The means and standard errors of eggs rayed 400, 500, and 600 r are shown in Curve 3. Curve 4 is of lots rayed 800, 1,000, 2,500, and 3,200 r and is drawn through the mean.

In these experiments dosages of over 800 r completely inhibited embryo formation in all eggs. The oxygen-consumption rate of such eggs (Curve 4) remained at about the initial level. It appears, therefore, that the increase in oxygen consumption during prediapause is dependent on the formation and activity of the embryo cells.

EFFECT ON INCREASE IN GROSS WEIGHT

It was observed that during the prediapause development the controls and some of the irradiated eggs gained in gross weight. This gain in weight is evidently due to the fact that eggs take up water. Slifer (1937, 1938) has recently reported observations and experiments on the development of the egg membranes and the water-absorbing area.

Some eggs were weighed at the end of the experiments (early diapause), and the results are shown in Figure 3. It was found that dosages below 800 r reduced the mean gain in weight and dosages above this amount prevented any gain at all. This finding indicates that complete inhibition of development prevents the increase in gross weight that one finds occurring in the prediapause development of the unirradiated eggs.

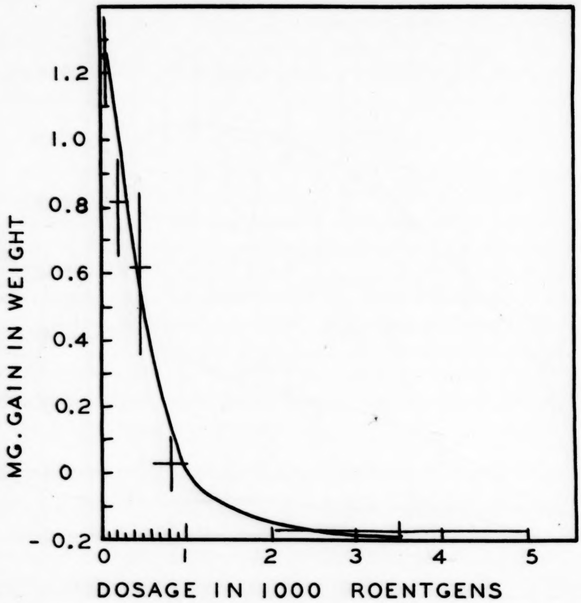

FIG. 3.—Means and standard errors of weights of controls and of 4 groups of irradiated eggs, at the end of the experiment, expressed as milligrams gain over that of the day irradiated. The initial weight was taken as 3.4 mg., which was the mean weight, on the first day, of 13 lots. The controls consisted of 19 eggs, and they all contained embryos. The 100–300 r group consisted of 14 eggs, and the 2 of this group which did not contain embryos had not gained in weight. Eight eggs were in the lots rayed with dosages of 400–500 r, and only 1 did not contain an embryo. The lots of 600–1,600 r consisted of 7, and none of the eggs contained an embryo. Those irradiated with dosages of 2,000–5,000 r were 8 in number, and none of these contained an embryo.

The relation between the increase in gross weight and the development of the embryo can be seen also by following the weights of control and irradiated eggs during the prediapause period. Curve 1 of Figure 4 shows that the weight increases rapidly after the embryo is formed (fifth to sixth days) and reaches the maximum at diapause. In an experiment in which eggs had been irradiated with a dosage of 500 r it became apparent by the tenth day that the eggs varied in size. From that time until the end of the experiment the eggs were selected for size, weighed individually, and then dissected. The weights of those eggs found to contain embryos are indicated in Curve 2 (solid squares). Curve 3 (open squares) is drawn through the weights of eggs which did not contain an embryo. It can be seen from an inspection of the graph that the weights of the eggs containing embryos were higher than those of eggs in which the embryo was missing.

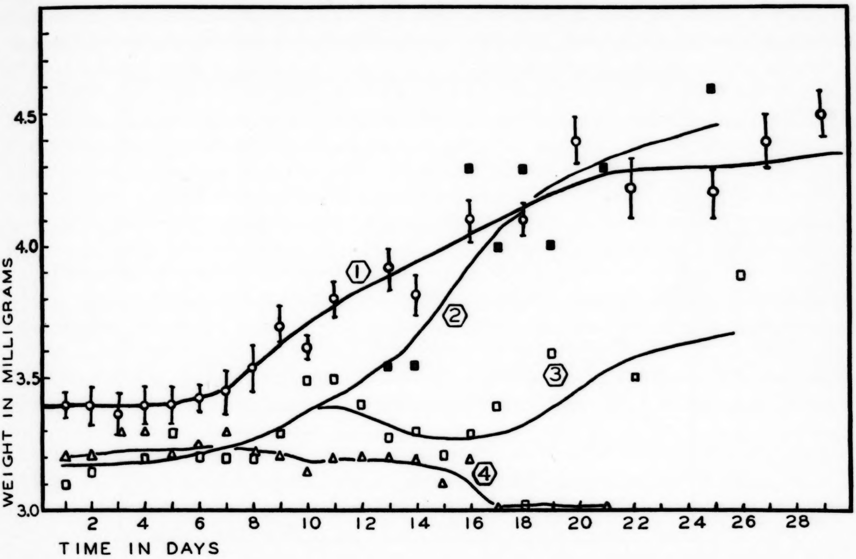

FIG. 4.—Curves showing effects of roentgen radiation on the subsequent weights of eggs during the prediapause period of development. Curve 1 shows the means and standard errors of 13 lots of control eggs. Curves 2 and 3 are of a lot of eggs rayed with a dosage of 500 r. From the tenth day the curves are distinguished on the basis of presence or absence of an embryo. Curve 2 (drawn through solid squares by inspection) is of eggs found to contain embryos. Curve 3 (through open squares) is of eggs in which embryo formation had been prevented. Curve 4 is of a lot of eggs irradiated with a dosage of 2,500 r. No embryos were found in any of these eggs.

One lot of eggs was irradiated with a dosage of 2,500 r and weighed daily for 21 days. The results are shown in Curve 4. The eggs failed to gain, and after 16 days they actually had decreased in weight. It is, therefore, concluded that, as in the case of oxygen-consumption rates, the increase usually seen in prediapause development is related to the development of the living constituents of the egg rather than to chemical or physical changes alone.

EFFECTS ON YOLK AND SEROSA NUCLEI

The curves of Figure 5 are survival curves in that they represent the number of yolk and serosa nuclei per egg when examined at the end of the experiment. The top curve, *A*, represents eggs between 2 and 12 hours of age when irradiated; the lower curve, *B*, eggs between 12 and 24 hours of age when treated. The eggs were examined when the controls were in early diapause. The number of yolk and serosa nuclei was determined by dissecting the egg in a drop of 5 per cent acetic acid, staining with Janus green, mounting in glycerine, and counting all the nuclei of the entire slide. Nuclei were counted instead of cells, as some of the cells contained more than 1 nucleus. The advantage of this kind of a preparation is in the ease and rapidity of the preparation with minimal loss of nuclei. These slides can be studied within 10 or 15 minutes and last very well for several days. In order to check the reliability of this procedure, one slide was counted 10 times and a mean value of 1,225 was obtained. The standard deviation of the mean was 77, and the standard error of the mean was 24.5, or 4 per cent. The smallest difference between irradiated and control eggs was about 25 per cent, so it appeared that this method of counting the nuclei was sufficiently accurate. The mean for the 55 control eggs was 873, with a standard error of the mean of 19.7. The results shown in Figure 5 indicate that the number of surviving yolk and serosa nuclei was reduced by the radiation. The survival data of the older eggs, which were plotted on semilogarithmic paper, fluctuated around a straight line, thus indicating that the dose-effect curve approximated an exponential one. The survival data for the younger eggs show too great an individual variation to place any interpretation on the character of the curve. The two curves are not significantly different, as the greater survival at 800 r, 1,600 r, and 2,000 r of the *A* curve over that of the *B* group was found to be only 0.265, 0.33, and 0.48 times the standard error of the difference. In the eggs rayed 2–12 hours after laying, the dosages of 1,600 r, 2,000 r, and 2,400 r resulted in reduction of survival numbers which were 1.48,

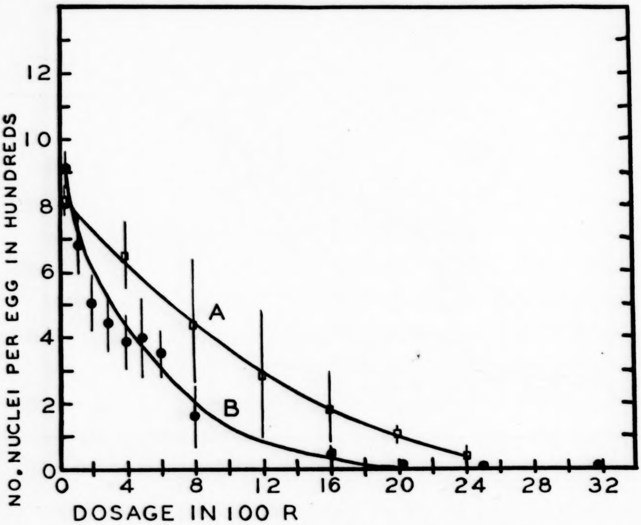

FIG. 5.—A graph showing the effect of roentgen radiation on decrease in the number of yolk and serosa nuclei surviving at diapause. The upper curve, *A*, is of eggs rayed when between 2 and 12 hours of age. The lower curve, *B*, is of eggs between 12 and 24 hours of age when irradiated.

2.4, and 3.4 times the standard error. Only the dosages of 2,000 r and 2,400 r reduced the number of surviving nuclei to a statistically significant degree. In the older eggs—12–24 hours of age—a dosage of 800 r produced a decrease which was 2.34 times the standard error. The decrease for the dosages of 1,600 r, 2,000 r, and 2,500 r were even more significant (2.6, 2.95, and 2.95 times the standard error).

An examination of Figure 6 will reveal three characteristics of the response of 1-day eggs to irradiation. In the first place, it is evident that the resistance of embryo formation varied in a marked degree. Some of the eggs irradiated with dosages as low as 200–400 r failed to form an embryo, whereas a few eggs were found to contain embryos even when irradiated with a dosage of over 1,000 r. Another characteristic of the effects on these eggs is the close relationship between presence of embryo and presence of serosa and yolk nuclei. It appears, especially in Group A, that the radiation either prevented formation of both embryo and yolk-serosa cells or allowed both to develop. In the *B* group there is some indication that the yolk and serosa cells were slightly more resistant than the embryos. A third observation to be made of the data in Figure 6 is that increasingly heavy dosages decreased the number of eggs containing the full complement of nuclei and increased the number which contained none at all rather than decreasing the number per egg. This is more evident in Group A than in Group B.

Five eggs of Group A were examined at the time they were irradiated, and the mean number of nuclei found was 1.6 ± 0.54. Several eggs have been examined between the twelfth and twenty-fourth hour, and the mean of this group was 9 ± 1.2 nuclei per egg. Slifer and King (1934) have sectioned *M. differentialis* eggs and have found eggs 9 hours after laying to have from 1 to 3 nuclei present. Eggs 1 day of age were found to have from 61 to 32 segmentation nuclei present. It was found in the present investigation that during the second day of development the number of cleavage nuclei increased rapidly. An egg examined at 10:00 A.M. on the second day contained 10 nuclei, at 2:00 P.M. another egg contained 36, and one egg examined at 5:30 P.M. was found to have 134 nuclei. During the third day, the number continued to increase rapidly, and counts of 183 and 269 were made. On the fourth day an egg was found to have 455 nuclei, which were very small at this time. On the fifth day the number of nuclei reached a maximum of 1,033. Cells of the embryo could easily be distinguished, and many of the others appeared to be differentiating into serosa cells. The nuclei then increased in size, and typical serosa and yolk cells were found in the microscopic preparations. In another lot of control eggs the yolk and serosa cells could be distinguished by the fourth day. In all cases the cleavage cells appeared to go through a period of rapid mitotic activity for 2 or 3 days, during which time the cells decreased in size. This was followed in the next few days by continued mitotic activity of the cells in the germ band, but those of the yolk and serosa ceased division, became large, and differentiated into an appearance typical of those of the rest of the diapause period. This early development presents many interesting phenomena, but interpretations should await further investigation.

Effects of radiation on the cells can be detected as early as the day following the treatment. The effects noted were (1) abnormal mitoses, (2) pyknosis, (3) phagocytosis, and (4) a reduction in number of nuclei as compared to the controls.

For purposes of clarity this discussion is limited to the number of surviving yolk and serosa nuclei (completely formed), so the effect of radiation on lowering the cell number is not evident until the fifth day when the cells would normally have assumed the typical condition.

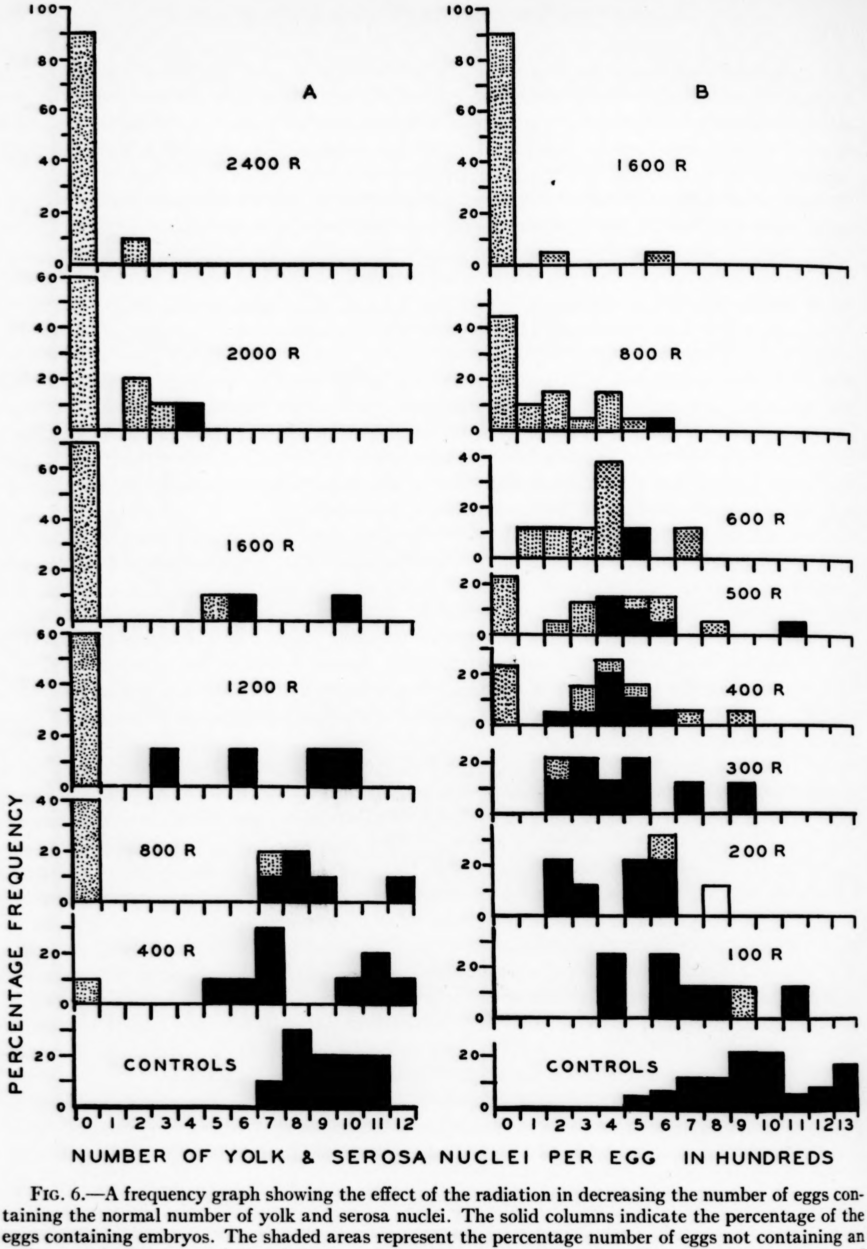

FIG. 6.—A frequency graph showing the effect of the radiation in decreasing the number of eggs containing the normal number of yolk and serosa nuclei. The solid columns indicate the percentage of the eggs containing embryos. The shaded areas represent the percentage number of eggs not containing an embryo. *A* is of eggs rayed when between 2 and 12 hours of age; *B* is of eggs between 12 and 24 hours of age when irradiated.

Typical serosa and yolk nuclei were formed between the fifth and seventh days in the control eggs as shown in Curve 1 of Figure 7. After a maximum of around a thousand nuclei was reached, the number remained about the same during the rest of the prediapause period.

One lot of eggs was irradiated with a dosage of 400 r, and the results are shown in Curve 2 of Figure 7. Individual variation was found, since some counts were as high as that of the controls whereas others were low. The general trend, as well as can be determined by inspection, seemed to indicate a slight delay in the attainment of the maximum number and for this peak to be a little lower than that of the controls. The effect of 500 r appeared to be more definite (Curve 3). In this case the maximum number was not reached until some 7 days after the controls, and the number of yolk and serosa nuclei finally formed was less than half of that of the controls. A dosage of 800 r (Curve 4) delayed the formation of the yolk and serosa nuclei still more and further reduced the number formed. Some eggs were irradiated with dosages of 1,600, 2,000, 2,500, and 3,200 r. With the exception of 1 egg (rayed 1,600 r), these failed to form any definite yolk and serosa nuclei. The eggs irradiated with dosages of 1,600 r and with 2,000 r were found at times to contain a few cells, but they were either undifferentiated or definitely injured.

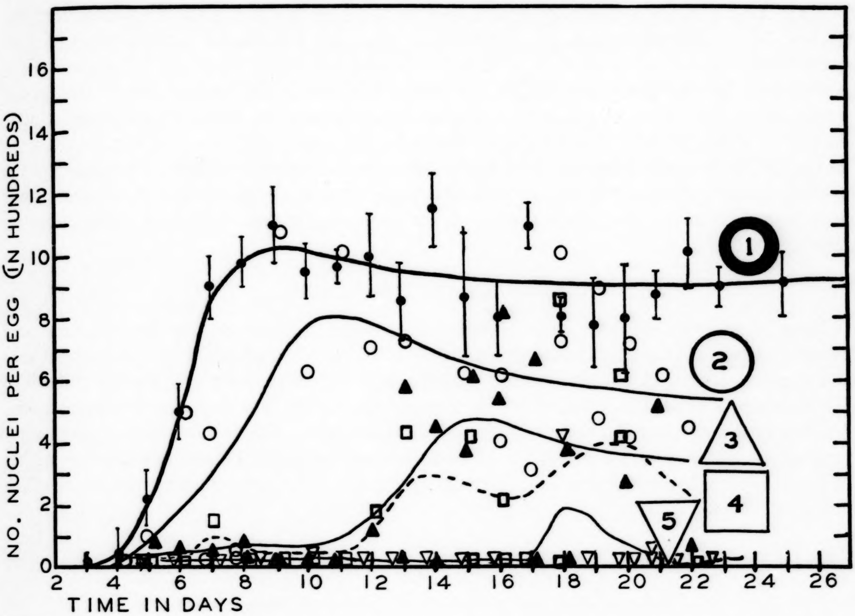

FIG. 7.—A graph showing changes in number of yolk and serosa nuclei of control and irradiated eggs. Curve 1 is through means and standard errors of 9 lots of control eggs. Curve 2 is of a lot of eggs irradiated with a dosage of 400 r. Curve 3 is of a lot of eggs rayed with a dosage of 500 r. Curve 4 is of eggs exposed to 800 r of radiation. A dosage of 1,600 r was given to the eggs of Curve 5.

DISCUSSION

The results indicate that irradiation on the first day may affect all the changes that normally take place during the prediapause development. These processes are dependent upon one another during the first day. This condition, if it occurs at all, must change very rapidly in *Drosophila*, since Henshaw (1935) states that mitosis goes forward apparently unmolested after irradiation, little or no differentiation takes place, and death is probably caused by unrestrained growth and lack of differentiation of the cells.

Ray (1938) has reported that irradiation on the first day prevents the subsequent formation of tyrosinase in the *M. differentialis* egg. He has found that dosages of 250–500 r lower the rate of tyrosinase production to such an extent that an additional 15 days is required for the concentration of the enzyme to reach the control level. Dosages of 1,000–2,000 r produce an inhibition of tyrosinase formation, from which there is little recovery. From these and other experiments he has concluded that the tyrosinase must be formed in the yolk or serosa cells rather than in the embryo proper. The results of the present investigation are in accord with his conclusion, as 1,000–2,000 r of radiation have been found to affect the development of the yolk and serosa cells with little or no recovery.

SUMMARY AND CONCLUSIONS

Eggs of *M. differentialis* have been irradiated on the first day of development and subsequent effects noted. The effects are failure to (1) form an embryo, (2) increase rate of oxygen consumption, (3) increase weight, and (4) form yolk and serosa cells. The developmental processes studied are closely related on the first day as a dosage of radiation heavy enough to affect one will also affect the others. The first effect to appear after irradiation is injury and destruction of undifferentiated cells. This effect appears within 24 hours after a heavy irradiation. The effects on the other processes appear on the fifth and sixth days.

LITERATURE CITED

BODINE, J. H. 1923. Hibernation in Orthoptera. I. Physiological changes during hibernation in certain Orthoptera. Jour. Exper. Zoöl., **37**:457–76.

———. 1929. Factors influencing the rate of respiratory metabolism of a developing egg (Orthoptera). Physiol. Zoöl., **2**:459–82.

———. 1932*a*. Hibernation and diapause in certain Orthoptera. II. Response to temperature during hibernation and diapause. *Ibid.*, **5**:538–48.

———. 1932*b*. Hibernation and diapause in certain Orthoptera. III. Diapause—a theory of its mechanism. *Ibid.*, pp. 549–54.

EVANS, T. C. 1934. Respiration and visible development of individual grasshopper eggs following x-irradiation. Physiol. Zoöl., **7**:556–71.

———. 1935. Variations in susceptibility to x-rays of *Melanoplus differentialis* eggs during development. *Ibid.*, **8**:521–29.

———. 1936*a*. Qualitative and quantitative changes in radiosensitivity of grasshopper eggs during early development. *Ibid.*, **9**:443–54.

———. 1936*b*. Use of roentgen radiation in analysis of metabolism in eggs of *Melanoplus differentialis*. Anat. Rec., Suppl. No. 1, **47**:60.

———. 1937. Effects of roentgen radiation on embryonic organization and regulation in *Melanoplus differentialis* (Orthoptera). Physiol. Zoöl., **10**:258–68.

———. 1938. Sequence of appearance of x-radiation effects. Anat. Rec., Suppl., **72**:43.

HENSHAW, P. S. 1935. Changes in susceptibility of *Drosophila* eggs to x-rays. II. Correlation of biological activity and radiosensitivity. Radiology, **24**:438–43.

RAY, O. M. 1938. Effects of roentgen rays on the activation and production of the enzyme tyrosinase in the insect egg (Orthoptera). Radiology, **31**:428–37.

SLIFER, E. H. 1932. External morphology of grasshopper embryos of known age and with a known developmental history. Jour. Morph., **53**:1–21.

——. 1937. Origin and fate of the membranes surrounding the grasshopper egg. Quart. Jour. Micr. Sci., **79**:493–506.

——. 1938. The formation and structure of a special water-absorbing area in the membranes covering the grasshopper egg. *Ibid.*, **80**:437–57.

SLIFER, E. H., and KING, R. L. 1934. Early stages in the development of grasshopper eggs of known age and with a known temperature history. Jour. Morph., **56**:593–601.

OXYGEN AS A FACTOR IN THE POLARITY OF CORYMORPHA PALMA

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BARTH (1938) has recently reported on the influence of oxygen in the reconstitution of *Tubularia*. The work of Child on the determination of polarity in cell aggregates (1928) and in stems of *Corymorpha* (1926, 1927) by the environmental differential between the upper free surface and the surface in contact strongly suggests that there also oxygen is a decisive factor. This presumption is supported by further work of Child and Hyman (1926), who found that respiration in *Corymorpha* is greatest in the hydranth and decreases basipetally over the naked stem region. Later Watanabe (1935) showed that the position of a future hydranth in a regenerating stem is marked out as a region of rapid methylene blue reduction several hours before the hydranth appears. In nature, too, where *Corymorpha* grows with its base imbedded in the mud and the hydranth extended freely into the water, it seems reasonable to assume the existence of a possible correlation between oxygen and polarized growth. In this paper I wish to present evidence that oxygen availability does actually affect hydranth formation and thereby influences the polarity of this organism. The work was suggested by Dr. Barth and was done during the summer of 1939 at the Scripps Institution of Oceanography. I am deeply indebted to the director, Dr. Harald U. Sverdrup, for the use of the laboratory; and to Dr. Marston C. Sargent and Mr. Percy S. Barnhart for many kind privileges.

METHOD

The specimens of *Corymorpha* used in these experiments were obtained during the "minus tides" of July at Mission Bay, where they may be found imbedded in the mud a few inches under water. They were about 3–4 cm. long. About 50 animals were obtained in all. The hydranths were cut off 1–2 mm. below the neck and discarded. Stem pieces averaging about 1.2 cm. in length were then snipped off 1–2 mm. above the perisarc-covered base. Basal as well as stem pieces were used. In the last experiment pieces were obtained from regenerated animals, since it was impossible to find fresh material. The temperature was approximately 21° C., except where otherwise stated below.

EXPERIMENTAL

Thirty-seven basal pieces were divided into two lots of 25 and 12, respectively. The first lot was allowed to regenerate in finger bowls of sea water. Within 24 hours they had healed at the cut surface and had secured themselves to the bottom of the dishes by means of holdfasts. Seventeen of the 25 had hydranths with rudimentary tentacles in 40 hours. Twenty-four had well-formed hydranths in 64 hours.

In the second lot the 12 bases were loosely capped over the cut surface with bits of glass tubing of $\frac{3}{32}$-inch bore, fused closed at one end. They were placed in finger bowls of sea water set on the sea-water table. These bases did not heal or regenerate at all during the course of the experiment, and later they disintegrated.

A procedure somewhat similar to the above was followed with 12 stem sections which were placed in pieces of submerged glass tubing of $\frac{3}{32}$-inch bore and $1\frac{1}{2}$ inches long, sealed at one end and loosely covered with cheesecloth at the other to prevent the stems from wandering out. These did not heal or regenerate. In comparison, 19 stems were divided into two lots of 10 and 9, respectively, and placed on cheesecloth stretchers in finger bowls of sea water. Flowing sea water, with oxygen content reduced to 3.71 cc. per liter, was siphoned into the dish containing 10 stems.[1] The water siphoned into the dish with 9 stems had a content of 5.07 cc. of oxygen per liter. In 21 hours at least two of the latter showed constriction of a hypostome. By 28 hours all of the stems at 5.07 cc. oxygen per liter and only half at 3.71 cc. had formed visible tentacle rudiments. In 34 hours 3 at 5.07 cc. and only one at 3.71 cc. had formed well-defined hydranths. At 44 hours fully developed hydranths with long tentacles and actively moving hypostomes were present in all at 5.07 cc. oxygen tension. This was true of only 5 out of 10 at an oxygen tension of 3.71. At 68 hours 3 out of the 10 in this lot still had hypostomes which were rather short and inactive.

In another experiment 12 stems were divided into two lots of 6 each and placed in glass-stoppered Erlenmeyer flasks of 125-cc. capacity. The flasks had been completely filled with aerated sea water, and nitrogen gas was bubbled through one for several minutes. Both were then sealed. By the end of 19 hours all the stems in the flask containing aerated sea water had healed and begun to form hypostomes. In 3 stems tentacle rudiments were present at 27 hours, and complete small hydranths were visible in 32 hours. One of these stems became bipolar during the next 10 hours. Compared with regeneration in normal sea water in the previous experiment, these show inhibition at this time, presumably due to consumption of oxygen by respiration. Between 42 and 52 hours, 2 additional stems had reconstituted, and, in all, 3 were bipolar and 2 unipolar. At 66 hours examination showed 5 out of 6 regenerated, but tentacles were still rudimentary in 2, indicating an insufficiency of oxygen for the completion of development. The flask was now opened, and the oxygen content was found to be 1.47 cc. per liter.

In the flask through which nitrogen had been bubbled the stems showed some healing at the ends but no further progress. When the experiment was terminated, chemical analysis showed that there was no free oxygen.

In a final experiment fragments were obtained from regenerated specimens. Four Erlenmeyer flasks were used. Nitrogen gas was bubbled through three of them for various lengths of time. Five basal and 4 stem pieces were placed in each flask. At 29 hours, owing to the clogging of the sea-water pipes, the temperature rose to, and remained at, about 26° C., and a few bubbles of gas appeared at the top of each flask. The results of this experiment must therefore be considered with caution. At 44 hours all the basal pieces had formed hydranths, whereas only 7 out of the total of 16 stems had begun to do so. All were retarded, in comparison with regeneration at the normal oxygen content of approximately 5 cc. per liter. At 52 hours all the basal pieces and 2 of the stems had fairly well-developed hydranths. The most significant difference was in the average number of tentacles developed by the basal pieces in the various flasks. These were 10.4, 8.4, 6.0, and 5.2, respectively, and were correlated with corresponding oxygen contents of 1.69, 1.75, 0.96, and 0.54 cc. per liter, determined at 68 hours when the flasks were opened.

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[1] The oxygen content of the sea water was reduced by bubbling nitrogen through a reservoir of slowly flowing sea water. I am indebted to Professor F. B. Sumner for this suggestion.

It may also be of some significance that the stem pieces were much more inhibited than the bases, indicating a possibly smaller oxygen requirement by the basal region.

DISCUSSION AND CONCLUSION

Although performed with a limited amount of material, the results of the above experiments all strongly suggest that oxygen is a determining factor in the formation of hydranths by regenerating portions of *Corymorpha* individuals.

It perhaps needs to be pointed out that bubbling nitrogen through sea water also probably reduces the CO_2 content. In connection with CO_2 variation, pH, too, may be affected. However, in the sealed flasks and in the glass tubes CO_2 is accumulated by metabolic activity. In this case pH (if affected beyond the buffering capacity of sea water at all) would also vary in a direction opposite to that in nitrogenated sea water. If metabolic products are presumed to play a part they, by the nature of the experimental procedures, would be accumulated in some cases and washed away in others. Therefore, while oxygen content was not the only variable in these experiments, it was the only factor with which hydranth formation can be consistently correlated.

As far as they go, these findings are in agreement with and support Barth's (1938) results with *Tubularia*. When considered together with evidence accumulating in other fields, such as the action of various growth-promoting substances upon root induction and the effects of CO_2, pH, etc., on polarity of *Fucus* (Whitaker, 1938), it seems reasonable to assume as a working hypothesis that, in organisms with labile polarity, pattern may be determined by factors which affect metabolic rate. Since Shapiro (1935) has demonstrated that *Arbacia* egg fragments containing the heavy granules have a higher respiratory rate than the lighter portions of the egg, it also becomes possible to interpret my results on the control of polarity in *Griffithsia* by centrifuging (Schechter, 1935) as a metabolic effect, i.e., as possibly due to higher respiratory rate at the centrifugal end. One is tempted to speculate further as to whether in organisms with fixed adult polarity the influences which emanate from organizing centers such as the lip of a blastopore during the labile period of embryonic development may not also be of a metabolic nature. Such an assumption would be supported by the multiplicity of agents which in experiments on induction have been shown to be able to act as organizers.

The evidence presented in this paper is in accord with Child's hypothesis of metabolic gradients. The experiments show that oxygen concentration influences the rate of hydranth formation. Since an existing hydranth exerts an inhibiting effect on hydranth formation by neighboring regions (Watanabe, 1935), the polarity of the animal is thereby determined.

SUMMARY

1. The regeneration of hydranths by sections of *Corymorpha* stem was noticeably inhibited at oxygen concentrations below 80 per cent of that of sea water which is in equilibrium with atmospheric air.

2. Retardation of oxygen diffusion by placing bases or stems in loosely fitting glass tubes submerged in sea water completely stopped reconstitution.

3. Sealing stems in comparatively large (125-cc.) volumes of sea water, from which most of the oxygen had been eliminated, completely stopped reconstitution.

LITERATURE CITED

BARTH, L. G. 1938. Oxygen as a controlling factor in the regeneration of *Tubularia*. Physiol. Zoöl., **11**: 179.

CHILD, C. M. 1926. Studies on the axial gradients in *Corymorpha palma*. III. Control and modification of polarity and symmetry in reconstitution by differential exposure. Biol. gen., **2**:771.

——. 1927. Modification of polarity and symmetry in *Corymorpha palma* by means of inhibiting conditions and differential exposure. Jour. Exper. Zoöl., **47**:343.

——. 1928. Axial development in aggregates of dissociated cells from *Corymorpha palma*. Physiol. Zoöl., **1**:419.

CHILD, C. M., and HYMAN, L. H. 1926. Studies on the axial gradients in *Corymorpha palma*. I. Respiratory, electric, and reconstitutional gradients. Biol. gen., **2**:355.

SCHECHTER, V. 1935. The effect of centrifuging on the polarity of an alga, *Griffithsia bornetiana*. Biol. Bull., **68**:172.

SHAPIRO, H. 1935. The respiration of fragments obtained by centrifuging the egg of the sea urchin, *Arbacia punctulata*. Jour. Cell. and Comp. Physiol., **6**:101.

WATANABE, Y. 1935. Physiological dominance in *Corymorpha palma* in relation to reconstitution and methylene blue reduction. Physiol. Zoöl., **8**:417.

WHITAKER, D. M. 1938. The effect of hydrogen ion concentration upon the induction of polarity in *Fucus* eggs. Jour. Gen. Physiol., **21**:833.

THE EFFECTS OF ACETYLCHOLINE, ATROPINE, AND NICOTINE ON THE ISOLATED HEART OF THE COMMERCIAL CRAB, CANCER MAGISTER DANA[1]

(Eight figures)

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THE effects of acetylcholine and of atropine on the arthropod heart have been studied by Welsh (1939*a*, 1939*b*) working with the hearts of *Carcinus* and *Panulirus*, by Hamilton (1939) working with the heart of the insect *Melanoplus*, and briefly by the writer and students (1940) working with the heart of the crayfish *Astacus*. Carlson (1906) nicotinized the heart of *Limulus*, in which ganglia and muscle can be separately treated. Yeager and Gahan (1937) and Yeager (1938) studied the effects of nicotine on the isolated hearts of the insects *Periplaneta* and *Prodenia*, Hamilton studied its effects on the grasshopper heart, and the writer made preliminary tests on the heart of the decapod *Astacus*.

There has been found to be considerable variation in the response of various arthropod hearts to acetylcholine; further information on its effect on the hearts of other forms as well as on its mode of action is needed. To the writer's knowledge no study has been made of the effects of nicotine on the decapod heart other than his brief tests mentioned above, which seemed to present evidence that the action of acetylcholine on crustacean hearts is "muscarine-like" rather than "nicotine-like." It seemed worth while, therefore, to carry on more extensive tests with these drugs. For this purpose the heart of the large commercial crab of the West coast, *Cancer magister*, was used.

The large size of this heart was found to be of advantage in making the perfusion studies. It was easily prepared for isolation and perfusion by simply cutting into the animal from the ventral surface after it had been allowed to bleed and removing musculature, digestive organs, and gonad. The heart could then be seen within the pericardium attached to the carapace by its ligaments. The carapace was then trimmed away leaving a small, easily manipulated section to which the heart was attached. Lastly, the pericardium was removed from the surface of the heart, and the heart flushed out with perfusion fluid via the aperture left by the removal of the descending vessel. If the heart had stopped during manipulation, flushing by this method and the consequent return of internal pressure quickly restored the normal beat. A small incision was made just proximal to one or the other of the ventral anterolateral alae cordis, the cannula inserted and tied in. A thread was tied around the posterior tip of the heart just anterior to the median posterior ala and including the posterior aorta. The heart was then suspended by a thread between cannula and writing-arm after the remaining ligaments and vessels had been cut.

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[1] The writer wishes to express his thanks to Dr. Frank Weymouth of Stanford University and Dr. John Welsh of Harvard University for aid and advice during this work.

For perfusion a simple constant-level chamber was designed, based, with modifications, on that used by Welsh (1939*a*) for the heart of *Carcinus*.

After some experimentation a perfusion fluid was arrived at which closely resembled that used by Pantin (1934) and by Welsh:

NaCl	0.53 M	1,000 cc.
KCl	0.53	25 cc.
$CaCl_2$	0.53	30 cc.
$MgCl_2$	0.53	25 cc.
$NaHCO_3$	1.00	2 cc.
Dextrose		1 gm.

Experiments were carried out in a controlled-temperature room with the temperature at 15° C.

ACTION OF ACETYLCHOLINE

It was determined, just as had been in the heart of *Astacus in situ*, that different hearts responded quite differently to the mediator. The threshold of sensitivity varied between 1:10,000,000 and 1:1,000,000 in most uneserinized hearts. Some hearts, however, showed no acceleration or increase in amplitude until concentrations as high as 1:100,000 were administered. Generally, acetylcholine in above-threshold concentrations produced a more or less marked increase in amplitude and frequency. If a heart had been beating irregularly, threshold concentrations regularized and slightly accelerated the rhythm. In certain hearts, however, acetylcholine produced no marked increase in frequency or amplitude but produced an increase in tonus which was reflected by a rise in level of the recording (Fig. 1). High concentrations had been shown by Welsh to produce in *Carcinus* such a rapid firing of the pacemaker cells that the heart was unable

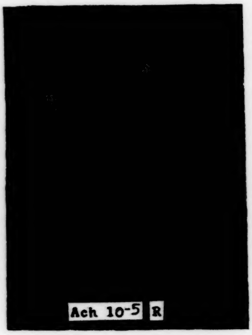

FIG. 1.—Effect of acetylcholine 1:100,000

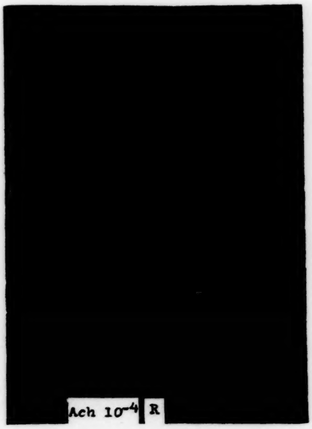

FIG. 2.—Effect of acetylcholine 1:10,000

to recover after each contraction, and a condition of systolic tetany followed. Such high concentrations as this were not tested on hearts which responded to low concentrations with the usual increase in frequency and amplitude, but in a heart which had responded by a tonus increase alone a solution of 1:10,000 produced such a marked increase in tone that the heart went into systolic stoppage. That this was not due to rapidly succeeding discharges from the pacemaker and was the result of a marked tonus increase can be seen in Figure 2.

As has been demonstrated in the hearts of other Crustacea, eserine increases the sensitivity of the heart of *Cancer* to acetylcholine; treatment with eserine 1:100,000 for 20 minutes would increase the sensitivity to the mediator from ten to one hundred times.

ACTION OF ATROPINE

This drug in concentrations as low as 1:20,000 abolished completely the effects of subsequently administered above-threshold concentrations of acetylcholine.

ACTION OF NICOTINE

Nicotine at a concentration of 1:20,000 produced in the isolated *Cancer* heart an increase in frequency and amplitude. This effect was not immediately reversible, for the heart would return approximately to its original beat only after some 15–20 minutes of washing with perfusion fluid.

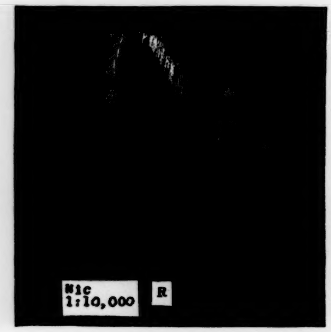

FIG. 3.—Effect of nicotine 1:10,000

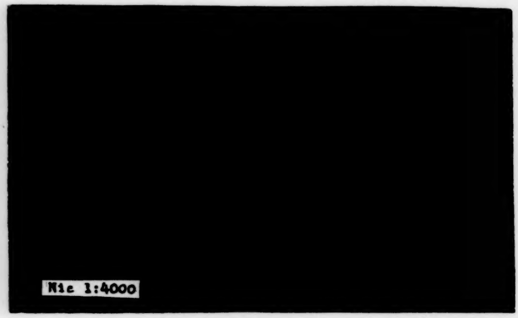

FIG. 4.—Effect of nicotine 1:4,000

The effect of nicotine 1:10,000 was interesting. In certain tests this concentration produced merely an increase in amplitude and frequency much as did nicotine 1:20,000. In other tests, however, it brought about systolic tetany after a brief and very marked increase in frequency accompanied by a rise in tone (Fig. 3). There was evidence in one such test that a certain "conditioning" to the drug existed, for a rapid and deep beat started again before the reintroduction of perfusion fluid into the heart.

Nicotine in higher concentrations (1:4,000, 1:3,000) had varying effects. In certain tests (Fig. 4) a rise in tone or an increase in amplitude and frequency was followed by a decrease in frequency or irregularity of beat. In other tests the depressant effects of such concentrations following an increase in frequency did not become evident, but in spite of 20 minutes of washing with perfusion fluid the amplitude steadily decreased.

In most such tests there appeared a transitory rise in tone; however, the beat would return after a time to the same level with a slightly decreased amplitude.

In all tests with nicotine the effects of the drug were found to be somewhat permanent; tests with higher concentrations on a heart which had previously been treated with lower concentrations or vice versa did not bring about the same effect as treatment of a fresh heart, since the effects were never completely reversible.

ACTION OF NICOTINE AND ACETYLCHOLINE

A heart was brought to systolic stoppage with nicotine 1:10,000; and the beat shortly thereafter, in spite of washing with perfusion fluid, steadily became weaker and slower (apparently because of the depressing effect of the nicotine). Treatment with acetylcholine 1:100,000 brought about a transitory increase in frequency and amplitude (Fig. 5). A number of hearts which had previously been treated with nicotine 1:4,000 responded to acetylcholine 1:100,000 with the usual increase in frequency and amplitude. In a test in which continuous treatment with nicotine 1:4,000 had produced a very irregular beat, acetylcholine 1:10,000 had a marked regularizing effect which was followed by stoppage (Fig. 6). The irregular beat returned after brief washing with perfusion fluid. A heart was treated with acetylcholine 1:100,000, which was found to bring about a rise in tone without an accompanying increase in frequency. Later treatment with nicotine 1:3,000, which brought about an increase in amplitude, did not prevent the appearance of the same rise in tone when the acetylcholine was reintroduced (Fig. 7).

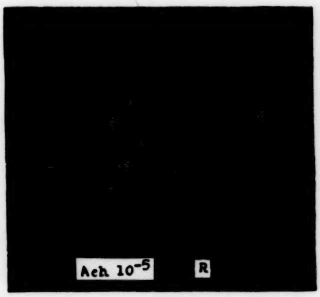

FIG. 5.—Effect of acetylcholine 1:100,000 after treatment with nicotine 1:10,000.

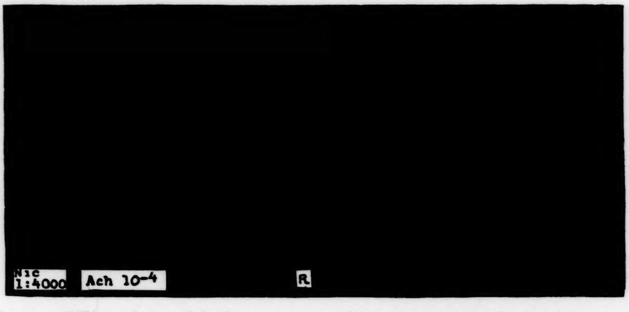

FIG. 6.—Effect of acetylcholine 1:10,000 after treatment with nicotine 1:4,000

DISCUSSION

The tests with acetylcholine which brought about a tonus change in the heart with no frequency increase would seem to indicate that the mediator works on the contractile mechanism as well as on the pacemaker and may work quite independently upon each.

But the effects on the contractile mechanism seem to become apparent only when high concentrations are used.

For some time there has been a question in the minds of workers as to whether the effect of acetylcholine on the arthropod heart is nicotine-like or muscarine-like. This writer has stated (1940) that the effects on the crayfish heart seemed to be muscarine-like since they were abolished by atropine; furthermore, there seemed to be no resemblance between the effects of nicotine and acetylcholine. But the nicotine tests on which these tentative opinions were based were brief and inconclusive. Not a wide enough variety of concentrations were used; and it is also questionable whether the drugs reached the intrinsic ganglion without dilution, since the heart was autoperfused. Nicotine 1 : 10,000 was found to bring about an increase in amplitude but a decrease in frequency. It is difficult to interpret these results in the light of those obtained on the isolated *Cancer* heart. It is possible that for the *Astacus* heart a concentration as high as 1 : 10,000 had a depressing effect on the pacemaker, but one would have expected an initial stimulation.

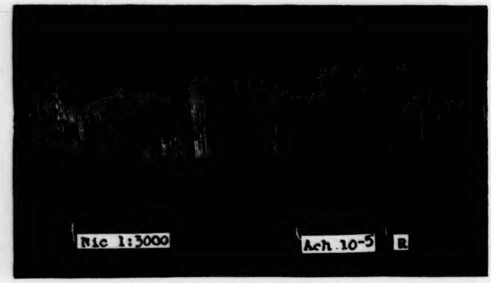

FIG. 7.—Effect of acetylcholine 1 : 100,000 after treatment with nicotine 1 : 3,000.

In the heart of *Cancer* it was found that the effect of acetylcholine is abolished by atropine. However, it was also found that nicotine 1 : 10,000 brings about systolic stoppage in the *Cancer* heart, at least for a brief period, by stimulating the pacemaker to discharge so fast that the muscle is unable to recover and systolic tetany results. This is strikingly similar to the response of the heart of *Panulirus* to acetylcholine 1 : 10,000 (Welsh, 1939*b*). Furthermore, in *Cancer* nicotine in lower concentrations causes a marked increase in frequency and amplitude just as does acetylcholine, though the effects of the former are somewhat cumulative and irreversible. There is evidence, therefore, that the two substances work in much the same manner on the isolated and perfused heart. Carlson (1906) in his classic experiments on the heart of *Limulus*, in which ganglion and muscle can be separately treated, was the first worker to demonstrate that nicotine in dilute solutions has a primary stimulating effect on the ganglion of an arthropod heart and that at higher concentrations the drug produces irregularity in rhythm, depression, and paralysis. He showed that in lower concentrations nicotine has little effect on the muscle itself. It seems probable, therefore, that in the crustacean heart perfused with nicotine the intrinsic ganglionic cells are stimulated.

The innervation of the hearts of various Crustacea has been extensively discussed by Alexandrowicz (1932). Welsh (1939*b*) says:

> Alexandrowicz has shown that, in spite of the small number of nervous elements, the morphological situation is likewise complex. Transection of the nerve trunk in the mid-region stops the regular beat of the heart. The ganglion may then be considered the pacemaker of the heart . . . . and . . . . any substance which (1) affected the pacemaker activity of the ganglion cells, (2) facilitated transmission at myoneural junctions, or (3) acted on muscle cells directly might modify the frequency and amplitude of beat.

TABLE 1

EFFECTS OF DRUGS ON ARTHROPOD HEARTS

Animal	Acetylcholine Threshold	Eserine	Acetylcholine after Atropine	Nicotine	Acetylcholine after Nicotine
Carcinus sp. (Welsh, 1939*a*)	10^{-8}	Not tested	Abolished		
Panulirus argus (Welsh, 1939*b*)	10^{-10} 10^{-9}	10^{-5} regularized beat or increased frequency; subsequent acetylcholine not tested	Abolished		
Limulus polyphemus (Carlson, 1906)				*Ganglia:* Low concentrations gave stimulation; high gave stimulation followed by depression and paralysis *Muscle:* Low concentrations had little effect	
Astacus trowbridgei (Davenport, Loomis, and Opler, 1940)	10^{-10}	10^{-5} regularized or gave initial increase in amplitude followed by decrease in amplitude and increase in frequency; subsequent acetylcholine not tested	Abolished	1:10,000 gave increase in amplitude and decrease in frequency; 1:5,000 decrease in amplitude and irregularity of beat	
Cancer magister (present work)	10^{-7}	Some hearts showed increase in frequency, some no effect; 10^{-5} for 20 minutes lowered threshold to acetylcholine approximately ten times	Abolished	1:20,000 gave increase in amplitude and frequency; 1:10,000 increase in amplitude and frequency or increase in frequency to systolic stoppage; 1:4,000, 3,000 initial stimulation of frequency and amplitude followed by depression	High concentrations do not abolish effect of subsequently administered acetylcholine
Melanoplus differentialis (Hamilton 1939)	1×10^{-14} M (2.26×10^{-15})	Not tested	Abolished	1:100 to 1:1,000,000 gave increase in amplitude and slight decrease in frequency; low concentrations in some hearts gave initial increase in frequency	

TABLE 1—*Continued*

Animal	Acetylcholine Threshold	Eserine	Acetylcholine after Atropine	Nicotine	Acetylcholine after Nicotine
Periplaneta americana and *Prodenia eridania* (Yaeger and Gahan, 1937)				Low concentrations gave initial stimulation in frequency without depression; intermediate concentrations, stimulation followed by partial depression; and high concentrations, stimulation followed by depression and paralysis; concentrations giving initial stimulation increased tone of heart	

A characteristic nicotine-like effect of acetylcholine in vertebrates is stimulation of the cells of sympathetic ganglia. There is evidence that acetylcholine has this stimulatory effect on the ganglion of the crustacean heart, because of the similarity of its effect to that of nicotine, which we know stimulates arthropod ganglionic cells. But in vertebrates muscarine-like effects are abolished by atropine, just as is the effect of acetylcholine on the *Cancer* heart.

It is possible to formulate a hypothesis to explain this anomolous situation in the *Cancer* heart. Acetylcholine may have a nicotine-like effect on the ganglion cells and may have a muscarine-like effect at the myoneural junction (Fig. 8). If, as in the vertebrates, nicotine-like effects are abolished by high concentrations of nicotine, then in the light of this "two-effect" hypothesis the stimulatory effects seen in Figure 5, in which the ganglionic cells have been depressed by high concentrations of nicotine, cannot be a result of stimulation of these cells but must be the effect of the introduced acetylcholine on the myoneural junction or the muscle directly.

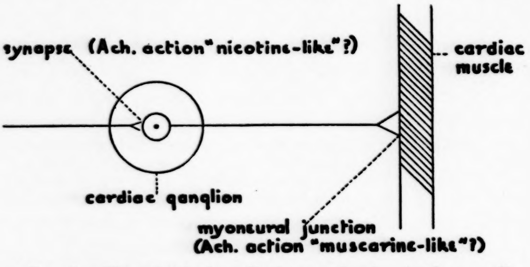
synapse (Ach. action "nicotine-like"?)
cardiac muscle
cardiac ganglion
myoneural junction
(Ach. action "muscarine-like"?)

FIG. 8.—Hypothetical mediation mechanism in the ganglion and muscle of the heart of *Cancer*.

It is apparent that further experiments must be carried out to test this hypothesis.

Table 1 presents a brief summary of the effects of the drugs discussed on arthropod hearts. It can be seen that the effect of nicotine on the hearts of *Periplaneta* and *Prodenia* (Yeager and Gahan, 1937) is somewhat similar to its effect on the heart of *Cancer*. However, in the roach heart systolic stoppage is brought about by nicotine by a steady increase in tone without a marked increase in frequency (Yeager, 1938, Fig. 7). It can be seen that in the heart of *Melanoplus* Hamilton (1939) noted little increase in frequency following treatment with nicotine, and that the heart of this insect is remarkably sensitive to acetylcholine.

LITERATURE CITED

ALEXANDROWICZ, J. S. 1932. The innervation of the heart of the Crustacea. I. Decapoda. Quart. Jour. Micro. Sci., **75**:181.

CARLSON, A. J. 1906. On the point of action of drugs on the heart with special reference to the heart of *Limulus*. Amer. Jour. Physiol., **17**:177.

DAVENPORT, D.; LOOMIS, J. W.; and OPLER, C. F. 1940. Notes on the pharmacology of the hearts of *Ariolimax columbianus* and *Astacus trowbridgei*. Biol. Bull., **79**:498.

HAMILTON, H. L. 1939. The action of acetylcholine, atropine, and nicotine on the heart of the grasshopper (*Melanoplus differentialis*). Jour. Cell. Comp. Physiol., **13**:91.

PANTIN, C. F. A. 1934. On the excitation of crustacean muscle. I. Jour. Exper. Biol., **11**:11.

WELSH, J. H. 1939*a*. Chemical mediation in Crustaceans. II. The action of acetylcholine in nervous tissues and its action on the decapod heart. Jour. Exper. Biol., **16**:198.

———. 1939*b*. Chemical mediation in Crustaceans. II. The action of acetylcholine and adrenalin on the isolated heart of *Panulirus argus*. Physiol. Zoöl., **12**:231.

YEAGER, J. F. 1938. Mechanographic method of recording insect cardiac activity, with reference to effect of nicotine on isolated heart preparations of *Periplaneta americana*. Jour. Agric. Res., **56**:267.

YEAGER, J. F., and GAHAN, J. B. 1937. Effects of the alkaloid nicotine on the rhythmicity of isolated heart preparations from *Periplaneta americana* and *Prodenia eridania*. Jour. Agric. Res., **55**:1.

OBSERVATIONS ON RODENTS IN HERTER'S TEMPERATURE GRADIENT

(One figure)

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HERTER described in 1924 a new temperature-gradient apparatus (*Temperaturorgel*) which was intended particularly for observations on the modal or so-called preference temperature of rodents. This new gradient differs from the older type used by the same author by having angled thermometers inserted within a bottom layer of aluminum. The readings thus obtained are obviously those of the bottom temperature. To correlate those results with the modal temperature of the rodents seemed justified, as these animals cling to the bottom with a very broad ventral surface.

Herter was not unaware of the fact that there exists a rather considerable difference between the temperature of the aluminum layer and the temperature of the air above it. But in his experiments the modal temperature of the rodents remained unchanged when the horizontal differences of air temperature were eliminated and equalized by ventilation of the interior of the gradient for short periods. In addition, mice with blackened belly (+) and those with depilated belly (−) showed an increase or decrease of about 1° C. in their modal temperature as compared with normal individuals. Herter, therefore, concluded that the mice are directed in their thermo-orientation only by the soil temperature.

According to our experiments on the scale of activity, the instantaneous death temperature of the recently born (up to a fortnight old) *Microtus guentheri* D. and A. fluctuates between 38.5° and 39.5° C. Whereas the modal temperature of the same ages of this vole, as it was measured by Herter's procedure, ranges between 38.5° and 39.0° C. The contradiction of these results is obvious and definitely excludes the assumptive identity of soil temperature and modal temperature.

A simple consideration may help to understand this contradiction. People in a room of 16°–20° C. like to press their backs to a stove of Dutch tiles with a surface temperature of 37°–41° C. This common experience would not make anyone assume that 37°–41° C. is the modal or preference temperature of *Homo sapiens* L. Despite the partially justified criticisms of Gunn, the readings of the air temperature at a distance from the soil which is in relation to the size and the height of the species studied are the relatively safest indicators for studies of modal temperature. This method was, therefore, tried in comparison with Herter's method.

The presence of air thermometers within the gradient interferes and disturbs the observations on the modality of the rodents. Air temperatures were therefore measured at different heights in the empty apparatus, whereby the agreement of the general course of temperature during the observation was ascertained by comparing the readings of the soil thermometers. The conversion table, calculated from these observations for an air temperature at 2.5 cm. above the soil, is shown graphically as Figure 1.

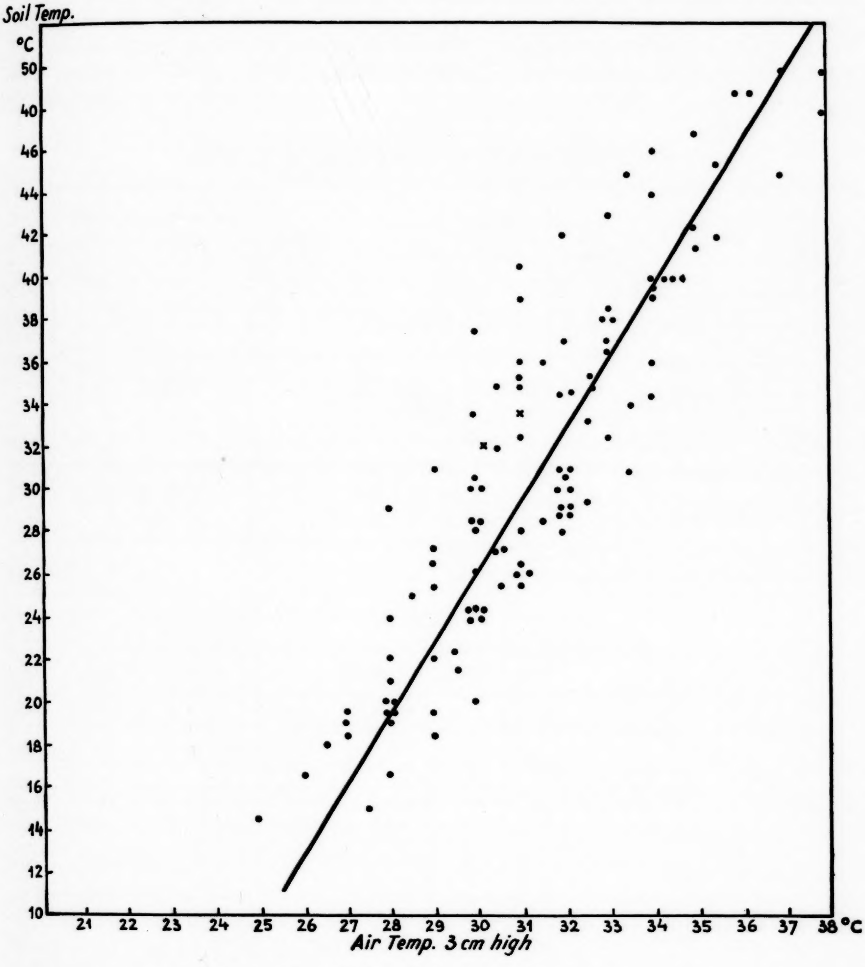

FIG. 1.—Conversion table of soil temperatures into air temperatures

EXPERIMENTS

All readings on the soil thermometers were converted by means of these tables. The results concerning the adults of 11 different species of rodents are compiled in Table 1 (M = statistical average; m = mean error).

The averages of the modal temperature of all these rodent species seem to be rather close, much closer than in Herter's publications. The analysis as to whether the differences are significant or not is made by the formula

$$x = \frac{M_1 - M_2}{\sqrt{(m_1)^2 + (m_2)^2}} \, .$$

TABLE 1

MODAL TEMPERATURE OF SOME RODENTS OF PALESTINE

No.	Species	Weight in Grams	*n*	*M* (Herter's Method)	*M* ± 3*m* (Our Method)	Temperature (° C.)												
						25	26	27	28	29	30	31	32	33	34	35	36	37
1...	*Cricetulus migratorius cinerascens* Wag.*	28–36	181	31.07	31.05±0.252	...	...	...	3	14	*36*	*61*	*53*	13	1	...	...	...
2...	*Cricetus auratus* Wtrh.†	66–85	253	32.73	31.51±0.243	...	...	...	3	8	*52*	*49*	*90*	*39*	9	3	...	...
3...	*Rattus norvegicus* Erxl. (white)†	117–183	252	33.20	31.70±0.303	...	...	3	5	13	*33*	*50*	*73*	*47*	17	10	1	...
4...	*Mus musculus musculus* L. (white)†	12–17	249	32.48	31.44±0.246	...	...	4	4	14	24	*56*	*112*	29	5	1	...	...
5...	*M. musculus gentilis* Brants	10–15	262	34.03	31.84±0.096	...	...	...	...	...	7	*56*	*172*	27	...	...	...	...
6...	*Acomys cahirinus dimidiatus* Cretzsch.	20–60	205	32.50	31.43±0.222	...	...	...	...	14	18	*56*	*96*	16	5	...	...	...
7...	*Dipodillus dasyurus* Wagn.	22	62	33.35	31.60±0.378	...	...	...	...	...	10	*17*	*24*	10	1	...	...	...
8...	*Meriones tamaricinus tristrami* Thos.	20–120	260	30.85	30.94±0.375	...	5	5	30	28	33	*36*	*61*	*43*	15	4	...	...
9...	*Microtus guentheri* D. and A.	15–80	398	31.66	31.23±0.267	...	...	11	23	25	*77*	*66*	*104*	*63*	27	4	2	...
10...	*Cavia cobaya* L.†	221–406	252	35.82	32.20±0.915	...	...	...	2	7	16	28	*77*	*84*	34	4	...	...
11...	*Spalax ehrenbergi* Nhrg.	....	134	32.04	31.28±0.294	...	...	...	...	5	*35*	*31*	*45*	16	2	...	...	...

* Experiments performed by Mr. Bindeman.

† Experiments performed by Mr. Wigodski.

TABLE 2

STATISTICAL ANALYSIS OF MODAL TEMPERATURES AS SHOWN IN TABLE 1*

Species Related	*x*	Species Related	*x*
5:1	9.3	10:1	8.3
5:2	3.7	10:2	5.0
5:3	1.2	10:3	3.3
5:4	4.5	10:4	5.5
5:6	5.1	10:5	3.1
5:7	1.9	10:6	5.8
5:8	6.9	10:7	3.6
5:9	6.6	10:8	7.6
5:10	3.1	10:9	6.8
5:11	5.4	10:11	2.8
Average	4.8	Average	5.2

* The value of *x* for the white rat and *Meriones*, e.g., is 4.7 (results are regarded as significantly different if *x* is larger than 3.0).

If *x* is larger than 3, the differences are regarded as statistically significant. The calculations given in Table 2 show that there is no doubt that most of the species have a significantly different specific modality. The recorded numbers for the different species are taken from the preceding table.

Three of the species used by us have also been studied by Herter. A comparison of the results obtained by both authors with Herter's method of calculation indicates a rather surprising degree of agreement for two species and a significant difference in the third one, as shown in Table 3.

These results show that Herter's new apparatus is quite reliable for obtaining comparable results in rodents. With regard to white mice the difference is possibly a constitutional one.

TABLE 3

COMPARISON OF TEMPERATURE MODALITIES WITH THOSE OBTAINED BY HERTER (1924)

Species	Herter's Results (° C.)	Our Results (° C.)
White rat....................	33.23	33.20
White mouse................	34.63	32.48
Cavia cobaya................	36.22	35.82

TABLE 4

MODAL TEMPERATURE OF *M. guentheri* AT DIFFERENT AGES

AGE IN DAYS	*n*	*M* (HERTER'S METHOD)	*M* ± 3*m* (OUR METHOD)	MODALITY AIR TEMPERATURE 2.5 CM. ABOVE SOIL (° C.)												
				27	28	29	30	31	32	33	34	35	36	37	38	39
2....	107	38.27	33.32±0.303	....	....	....	....	4	19	*37*	*34*	12	1	....	....	....
4....	113	38.52	33.21±0.243	....	....	....	....	3	15	*58*	*29*	8	....	....	....	....
6....	118	38.69	32.94±0.429	....	....	....	2	4	*25*	*47*	*27*	10	4	1	....	....
10....	164	37.81	35.00±0.336	....	....	1	4	4	12	5	9	*49*	*64*	15	1	....
15....	135	36.54	34.61±0.339	....	....	....	1	....	2	8	*57*	*61*	14	3	....	....
21....	128	33.28	33.67±0.342	....	....	....	....	8	21	14	*56*	21	7	1	....	....
40....	157	33.23	33.71±0.342	....	....	....	....	5	*33*	*29*	*47*	*30*	5	7	1	....
73....	142	32.12	33.31±0.426	....	....	1	5	18	25	19	*40*	23	8	1	2	....
81....	186	35.17	34.24±0.315	....	....	....	....	5	17	*31*	*55*	*46*	18	13	1	....
105....	118	30.72	30.93±0.477	1	10	10	24	21	*37*	10	3	2	....	....	....	....
209....	136	32.79	31.63±0.471	2	8	8	10	19	*31*	*32*	13	2	2	....	....	....
300....	144	31.48	31.03±0.426	8	5	7	*34*	*26*	*36*	21	7	....	....	....	....	....

The difference between the modality of young and old rodents is greatly reduced by the conversion of the soil temperatures into the air temperatures. Extensive experiments on *M. guentheri* yielded the results listed in Table 4.

Herter's statement that the modality decreases from birth to old age is confirmed in principle. The difference is, however, reduced from 8.15° C. (38.37°–30.72° C.) to 2.35° C. only (33.82°–31.47° C.). The higher modality between 10 and 15 days of age, i.e., shortly before and after weaning, may be caused either by the increased locomotor ability or by a lower degree of thermo-orientation in the neonatal rodents.

The difference between young and recently matured adults is small and insignificant, whereas the difference between either or both groups and old adults is highly significant, as shown in Table 5.

DISCUSSION

There is no doubt, as has been pointed out, that the results obtained with mammals in Herter's modified apparatus have no direct relation to the specific "comfortable" temperature. But, on the other hand, correlation between air temperature and modality yields principally identical results. The results obtained for 11 species of rodents are, practically, all significantly different with regard to their temperature modality. And this in spite of the fact that the range is much narrower than in Herter's method, where the readings are correlated to soil temperature.

The theoretical background is as follows: When poikilothermic animals are exposed to a temperature gradient, the body temperature and the intensity of all physiological processes are changed. No thermic sense organs are known, but it is reasonable to assume that the modal temperature is the so-called comfortable temperature. We are, unfortunately, unable, so far, to understand the mechanism of thermo-orientation in poikilothermic animals. There seems a possibility of connecting it, partially at least, with the water metabolism of the body tissues (Bodenheimer, 1931).

TABLE 5

STATISTICAL ANALYSIS OF THE EFFECT OF AGE ON MODAL TEMPERATURE OF *M. guentheri*

Age in Days	$M \pm 3m$ (° C.)	$\frac{M_1 - M_2}{m \text{ Diff.}}$
1–10	33.82 ± 0.201	1:2= 1.23
15–81	33.92 ± 0.132	1:3=23.3
105–300	31.22 ± 0.264	2:3=27.3

The thermo-orientation of mammals seems to be of an entirely different character. The more or less constant body temperature of homiothermic animals is a very unreliable guide, as we know from daily experience. Our thermo-orientation is highly influenced by contrast effect. Mammals possess poor powers of estimating surrounding temperatures. They can distinguish and avoid extreme temperatures and come to rest in the least stimulating of available temperatures. With man this zone of comfort fluctuates between 16° and 20° C. But this "comfortable" temperature is not the temperature obtained in Herter's apparatus. The rodents dilate the belly as far as possible and press it flat on the surface of the soil, just as men press the back against a stove with a surface temperature twice as high as the comfortable temperature, one entirely unfit for permanent sojourn. This is confirmed by our observation that the modality obtained in the temperature gradient for young *Microtus* is identical with their instantaneous death temperature.

What the experiment really proves is (1) the presence of thermo-orientation in rodents and (2) significant specific differences in the rodents studied. Herter's method of using the readings of the soil temperature is preferable to the conversion of these readings into air temperatures because (1) the directive temperature stimulus comes from the soil surface and not from the air temperature and (2) the range of fluctuation is larger for soil temperatures than for air temperatures and the readings therefore more convenient. Since this paper was written, a study by N. Kalabuchov (1939) has appeared, from which further details can be learned.

Our experiments were performed in an apparatus which was built exactly after Herter's indications. But Kalabuchov changed its proportions (length, 120 cm. versus 60 cm.; breadth, 15 cm. versus 10 cm.). This apparatus seems better suited to observations of rodents than does the rather short apparatus used by Herter and by myself. But, un-

TABLE 6

EFFECT OF LATITUDE AS MODAL REACTION IN A TEMPERATURE GRADIENT

Localities	No. of Specimens	No. of Readings	Modal Temperature (° C.)
Charkov	18	900	23.24
Moscow	17	850	26.44
Rostov	11	550	27.82
Pre-Caucasus	10	500	29.17

TABLE 7

MODAL TEMPERATURES FOR RODENTS AS DETERMINED TO DATE

SPECIES OF RODENTS	MODAL TEMPERATURES AS DETERMINED BY—		
	Herter	Bodenheimer	Kalabuchov
Eliomys quercinus	39.02±0.67		
Muscardinus pulcher	38.40±0.41		
M. avellanarius	38.45±0.29		
Glis glis	36.35±0.29		
Citellus pygmaeus			30.85
C. suslica			31.41
Cricetus cricetus	29.41±0.31		
C. auratus		32.73	
Cricetulus migratorius		31.07	
Mus musculus musculus	37.36±0.12		
M. m. musculus (white)	34.63±0.12	32.48	
M. m. gentilis		34.03	
M. m. azoricus	37.43±0.22		
M. wagneri rotans	33.85±0.13		
M. spicilegus	35.54±0.16		
Rattus norvegicus	35.06±0.18		
R. norvegicus (white)	37.23±0.27	33.20	
Micromys minutus	37.02±0.24		
Apodemus agrarius	36.14±0.22		
A. flavicollis	33.76±0.20		20.54
A. sylvestris	34.64±0.19		26.67
Evotomys glareolus	32.32±0.20		
Acomys dimidiatus		32.50	
Diplodillus dasyurus		33.25	
Meriones tristrami		30.85	
Microtus arvalis	35.05±0.30		
M. guentheri		31.66	
Arvicola terrestris	28.15±0.45		
Pitymys savii	29.39±0.43		
Cavia cobaya	36.22±0.19	35.82	
Spalax ehrenbergi		32.04	

fortunately, this modified apparatus gives entirely different results. This may be learned from the values obtained on two species of *Apodemus*, studied by both Herter and Kalabuchov. It seems, therefore, highly desirable, in order to obtain comparable results, that all students agree on one type of temperature gradient.

Kalabuchov makes the very interesting statement that the modal temperature increased in specimens of *A. silvaticus* L. from different latitudes, increasing from north to south, as shown in Table 6. The differences for another species (*A. flavicollis* Melch.) from Leningrad and Charkov were slight (only 20.34° as compared with 20.73° C.). Similar observations on poikilothermic animals have been made by Krumbiegel (1932, *Carabus*) and Lütfi (1936, *Lacertilia*). On the other hand, the three species of rodents (all domesticated species) studied both by Herter and by myself show a close agreement. This agreement is more remarkable as the climates of Berlin and Jerusalem differ more widely than those of the Russian localities among themselves. It is very desirable that such experiments should be continued with identical apparatus, in order to make certain that the results obtained are strictly comparable. Table 7 compiles the experimental results, so far obtained, on the modal temperature of rodents.

CONCLUSIONS

1. Observations of rodents in a temperature gradient show a distinct and specific thermo-orientation.
2. This thermo-orientation is directed by the soil temperature.
3. The modal temperature of rodents, as obtained in the temperature gradient, is not comparable with results obtained by the same method for poikilothermic animals. It is about twice as high as the "comfortable" temperature of each species.
4. For orientation on the specific differences of the thermo-orientation in different rodents the observation in the temperature gradient gives satisfactory results. No further conclusion should be drawn, and no value ascribed to the absolute temperatures of the readings. The latter may even approach or reach the temperature zone of instantaneous death.
5. The comparison between readings of soil temperatures and air temperatures show that both give useful results. For the sake of simplicity, readings of the soil temperature are preferable.
6. The importance of working with identical apparatus, in order to get comparable results, is pointed out.
7. The decrease of the modal temperature after birth is confirmed.
8. The problem of a geographical shift of the modal temperature of rodents within the same species is discussed.
9. All modal, or so-called preference, temperatures so far obtained for different species and varieties of rodents are compiled.

LITERATURE CITED

BODENHEIMER, F. S. 1931. Über die Temperaturabhaengigkeiten von Insekten. III. Zeitschr. f. vergl. Physiol., **13**:740–47.

GUNN, D. L. 1934. The temperature and humidity relations of the cockroach (*Blattella orientalis*). II. Zeitschr. f. vergl. Physiol., **20**:617–25.

HERTER, K. 1934. Biol. Zentralbl., **54**:487–507.

———. 1936. Zeitschr. f. vergl. Physiol., **23**:605–50.

KALABUCHOV, N. 1939. Zool. Jurnal (Russian), **18**:915–23.

KRUMBIEGEL, I. 1932. Zool. Jahrb. Syst. **63**:131–280.

LÜTFI, M. 1936. Diss. Naturwiss. math. Fak. Berlin.

THE EFFECT OF CYANIDE ON OXYGEN CONSUMPTION OF COLPIDIUM CAMPYLUM[1]

(Two text figures)

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BEGINNING with the work of Lund (1918), oxygen consumption of ciliates has repeatedly been found to be relatively unaffected by cyanide. This is of considerable general interest, since it suggests that the mechanism by which the infusoria utilize molecular oxygen is essentially different from the usual type occurring in aerobic cells.

Loeb and Wasteneys (1913) and Warburg (1914) first demonstrated that the toxic action of cyanide on living matter is due to its specific power of inhibiting intracellular oxidations. Subsequently, it has been shown that cyanide influences the first step in a chain of reactions by which hydrogen is removed from cell metabolites and transferred to molecular oxygen. Reduction of oxygen is accompanied by oxidation of cytochrome—an intracellular hydrogen-carrier complex. This step is catalyzed by cytochrome oxidase —an enzyme which is completely inactivated in the presence of cyanide in low concentrations. Almost the entire respiration of yeast is thus inhibited. With other types of cells, notably metazoan tissue cells, it was formerly supposed that a considerable and rather variable proportion of their oxygen uptake was cyanostable and hence could not involve the cytochrome-oxidase system (Dixon and Elliott, 1929). Recently, however, van Heyningen has shown (1935) that this conclusion was based upon the results of experiments performed under improperly controlled conditions. Employing an improved technique devised by Krebs (1935), he was able to show that about 80 per cent of the oxygen consumption of tissue cells is inhibited by 0.001 M cyanide, the degree of inhibition depending directly upon the concentration of the inhibiting agent. The evidence is conclusive that the mechanism involving cytochrome and its oxidase accounts for the major portion of oxygen utilization by most aerobic cells.

In the light of van Heyningen's findings with tissue cells it seemed worth while to reinvestigate the effect of cyanide upon oxygen consumption of a species of ciliate, adopting the method devised by Krebs (1935) to eliminate the errors arising from the properties of hydrogen cyanide. The holotrich *Colpidium campylum* was chosen for the purposes of this study because bacteria-free cultures of this species were available. In testing the effect of cyanide the potassium salt was used. The addition of KCN, even in low concentrations (0.0001–0.01 M), produces a shift in the pH of a buffered suspension of protozoa, the extent of the change depending upon the amount of KCN added, the nature of the buffer, and the initial pH. From the work of Sørensen (1909), Michaelis and Davidsohn (1911), and later investigators the sensitivity of enzyme systems to changes in H-ion concentration is now well known. It seemed advisable, therefore, as a corollary to the experiments with cyanide, to determine the relationship between oxygen consumption of *C. campylum* and the pH of the suspension.

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[1] Submitted, with the preceding paper on "The Oxygen Consumption of *Colpidium campylum*" (Hall, 1938), in partial fulfilment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University.

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MATERIAL AND METHODS

Cultures of *C. campylum* were maintained, bacteria free, in 20×150-mm., cotton-plugged, Pyrex tubes containing 15 cc. of sterile medium. Transfers were made with sterile 1-ml. pipettes, observing the usual precautions in order to prevent bacterial contamination. The medium employed for cultivation of the organisms consisted of a 2 per cent solution of tryptone (Difco), containing 0.1 per cent yeast extract (Difco). The nutrient ingredients were added to a standard buffer solution containing 0.1 gm. $MgSO_4 \cdot 8H_2O$, and 0.1 gm. $Ca(NO_3)_2 \cdot 4H_2O$. The buffers employed were Sørensen's phosphate mixtures ($M/15$ KH_2PO_4-Na_2HPO_4) and Clark and Lubs's biphthalate (0.05 M $KHC_8H_4O_4$-NaOH). The preparation of these buffers is described in detail by Clark (1928), with data for making up solutions of known pH. The initial pH of the suspensions was determined upon a sample taken at the beginning of every experiment. In addition the final pH of the contents of the manometer vessels was measured. The pH-measurements were made with a quinhydrone electrode connected through a saturated KCl-agar bridge with a saturated KCl-calomel half-cell.

The suspensions employed in the experiments were made up from cultures freshly inoculated 48 hours before they were to be used. The cultures were kept in the laboratory except during the summer months, when the temperature became too high. During this period—from June to October—the cultures were removed to a thermostat-controlled room at 60°–65° F. The technique of preparing the experimental suspensions and of determining their density has been described in detail in a previous paper (Hall, 1938), hence it need not be repeated here.

The cyanide solutions employed in the experiments dealing with the effect of this reagent were prepared from a molar solution of KCN by diluting with fresh culture medium of the same pH as the experimental suspensions. A measured volume of a suitable dilution of 1M KCN was placed in the onset chamber of the manometer vessel and was tipped into the main chamber after a 30-minute preliminary run. The volume and concentration were adjusted so that the desired final concentration of cyanide would be approximated in the experimental suspensions.

Oxygen consumption was measured directly by means of Warburg manometers. The details of the application of the method to the present problem were stated in the earlier paper referred to above (Hall, 1938). In the current research, manometer vessels of slightly greater capacity were used, averaging about 12.5 cc. in volume. The manometers were shaken continuously at the rate of 140 cycles per minute through an arc of 5 cm. In determining the relationship between oxygen consumption and the pH of the medium, three or four manometers in addition to a thermobarometric control were used in each run, and the results were averaged. In the experiments dealing with the effect of cyanide separate controls, together with control thermobarometers, were run simultaneously with the experimental manometers and thermobarometers.

EXPERIMENTAL RESULTS

"NORMAL" OXYGEN UPTAKE

In the author's previous paper (Hall, 1938) a value of 112.5 $mm.^3$ $O_2/hr./10^6$ was reported for the rate of oxygen consumption of *C. campylum* at 19.8° C. and pH 6.6. This figure was obtained by averaging the results of thirty-six measurements upon suspensions of ciliates in the medium in which they had been growing for 48 hours. With proper thermobarometric control the rate was found to be constant during a period of over 4 hours.

The data of these experiments provided an index of the accuracy with which samples of approximately the same density can be obtained from the original concentrated suspension using the technique described. The standard deviation of the measurements was 0.0363. For different samples from the same suspension, however, the standard deviation of the Q_{O_2}-values obtained was 0.0194. The combined error of sampling and counting, determined from the differences between counts performed on different aliquots from the same suspension, was about 4 per cent. Hence, the sampling error was well within the limits of error of the general method of relating the volume of oxygen consumed to the magnitude of the respiring population.

EFFECT OF pH

Table 1 is a summary of results of the experiments relating oxygen consumption of *C. campylum* to the pH of the medium in which the organisms were suspended. The pH given is the initial value to the nearest 0.1. Since deviations from a constant rate of oxygen uptake were slight with both of the buffers employed, the rates were calculated from the slope of the straight line which best fitted the data of each experiment. The values given in the table represent averages of the data obtained with each of the several manometers in a single run. Comparison of the results obtained in different runs performed on suspensions at the same initial pH leaves little doubt regarding the significance of the differences between rates observed at different initial pH.

The data indicate that there is a definite optimum H-ion concentration for oxygen consumption of *C. campylum* at about 2.82×10^{-6} (pH 5.55). On the alkaline side of this optimum, as the pH increases, the rate of oxygen consumption falls off gradually to about half the maximum at pH 7.65. Below pH 5.5 the rate of oxygen uptake decreases rapidly with increasing acidity.

The data in the third column of the table, indicating the magnitude and direction of the pH change which occurred in the course of each run, are particularly interesting. The greatest changes might have been expected to take place at the extremes of the pH range appropriate to the buffers employed. Inspection of the table shows that this was not the result observed. On the alkaline side of pH 5.5 the change was in the direction of increased alkalinity, and its magnitude increased as the pH; on the acid side the magnitude of the change was less, and its direction was reversed, increasing with increased acidity. The explanation of these phenomena is uncertain. In two instances with phosphate buffers (marked with an asterisk), the shift was so great in comparison with that observed in other experiments at the same initial pH that it seemed probable it was caused by creeping of the alkali in the inset chambers of the manometers into the main chambers. The results of these experiments were disregarded in subsequent analysis of the data. In all the other runs the pH change was too small to have been caused by alkali-creep. Moreover, in the suspensions buffered with biphthalate at pH below 5.5 the change was in the wrong direction to warrant such an explanation.

EFFECT OF KCN

The majority of investigators who have studied the effect of cyanide on the respiration of ciliates have found that there is an initial inhibition followed by rapid recovery and even by a period during which the respiratory rate exceeds the normal. The best studies are those of Pitts (1932) and M. Lwoff (1934). The former found that KCN in 0.01–0.001 M concentrations regularly produced a 20 per cent inhibition of oxygen consumption immediately upon introduction into a suspension of *C. campylum*. With the passage of time, however, the rate of oxygen uptake gradually increased until it surpassed the normal value. The length of time required to attain the maximum rate increased as the

TABLE 1

OXYGEN CONSUMPTION OF *C. campylum* IN RELATION TO THE pH OF THE SUSPENSION

Buffer	Initial pH	ΔpH	Q_{O_2}
Phosphate	7.5	+0.12	59.6
		+0.20	66.0
	7.4	+0.29	62.8
		+0.27	62.1
	6.8	+0.20	103.3
		+0.20	103.6
		+0.16	109.0
		+0.22	98.6
		+0.18	103.9
		+0.24	97.7
	6.4	+0.06	121.0
		+0.07	118.0
		+0.44*	106.0*
	6.3	+0.09	114.0
		+0.12	115.0
	5.5	+0.07	130.0
		+0.04	124.0
		+0.05	126.0
		+0.09	124.5
		+0.07	122.3
	5.4	+1.40*	106.5*
	5.3	+0.03	115.0
		+0.06	114.0
		+0.06	120.0
Biphthalate	5.9	0.0	125.5
		+0.02	125.5
		0.0	119.0
		0.0	123.3
		0.0	120.7
	5.4	−0.04	119.1
		−0.02	124.3
		0.0	121.6
	5.2	−0.03	86.3
		−0.03	84.5
		−0.05	86.5
		−0.04	92.0

concentration of the inhibiting agent. Lwoff secured analogous results with *Glaucoma pyriformis*. She found, however, that the initial inhibition in the presence of 0.001–0.002 M KCN might reach 80 per cent during the first half-hour.

These investigators tested the effect of cyanide by opening the manometers and adding KCN to the desired concentration after a preliminary run to establish the normal rate of oxygen consumption. Their cyanide solutions were prepared from concentrated stock solutions by "neutralizing" with a strong acid and diluting with the buffer solution in which the protozoa were suspended. The CO_2 formed in respiration was absorbed by means of a strong solution of KOH in the inset vessel of the manometers.

In the current research, experiments similar to those performed by Pitts (1932) and M. Lwoff (1934) were carried out with suspensions of *C. campylum*. The manometers were not opened during the course of the runs, however, for the purpose of introducing the cyanide, and the latter was not "neutralized" with strong acid. Typical curves for different concentrations of KCN are shown in Figure 1. The diagonal represents the average rate of oxygen consumption in the absence of cyanide, calculated from the data of the controls. The initial pH was 7.6. The black triangle on the time axis indicates the point at which the KCN solution in the onset chamber was tipped into the main chamber of the manometer vessels. The concentrations of cyanide given are approximately correct. The amount of KCN which is tipped into the suspension cannot be controlled exactly because of the fact that enough solution always remains in the onset chamber to wet the walls.

It will be observed that there is a marked initial inhibition of oxygen consumption, evident even before the KCN is tipped into the suspension. With 0.001 M KCN after 1 hour the rate of oxygen uptake surpasses that of the controls. Recovery is more gradual in the presence of higher concentrations of cyanide. In the presence of 0.005 M KCN the rate of oxygen consumption after 1 hour is about equivalent to that of the controls, while after the same interval there is still considerable inhibition with 0.01 M KCN. The data conform to the sort of curve described by Pitts (1932) and M. Lwoff (1934). This is the type which would be expected from measurements with steadily decreasing concentrations of inhibiting agent. It seems highly probable that the recovery noted after an initial inhibition of oxygen consumption is due to the disappearance of cyanide from the suspensions. The chemical properties of cyanides lend support to this conclusion.

In dilute aqueous solutions KCN reacts with water to yield KOH and HCN. The latter is a very weak acid and is extremely volatile. Hence the addition of a moderately strong acid to a solution of KCN merely serves to drive off HCN. This gas, like carbon dioxide, is readily absorbed by a strongly alkaline solution. In dilute aqueous media CO_2 dissolves to form H_2CO_3. This acid has a primary dissociation constant of 3.5×10^{-7}. The dissociation constant of HCN is 1.3×10^{-9}. In equivalent concentrations H_2CO_3 evidently will furnish approximately two hundred and seventy times as many hydrogen ions to a solution as will HCN. Thus, when cells are suspended in a buffered solution containing cyanide and are permitted to respire in a closed system (such as one of the manometers in the experiments described above), HCN will tend to distil over from the suspension into the KOH provided to absorb the respiratory CO_2. Increased glycolysis during cyanide inhibition tends to increase the rate at which the HCN distils off the suspension, since lactic acid is an even "stronger" acid ($k_a = 1.38 \times 10^{-4}$) than carbonic. As the amount of cyanide in the suspension decreases, the rate of respiration of the cells will increase; the increased CO_2-output of the cells will accelerate the disappearance of HCN until, at equilibrium, the concentration of the inhibitory agent remaining in the suspension will be negligible.

Krebs (1935) and van Heyningen (1935) have shown that the errors arising from the tendency for HCN to distil over into the CO_2-absorbing medium may be eliminated by replacing the KOH solutions usually employed for this purpose with KCN-KOH mixtures in which the concentration of free HCN is approximately the same as in the experimental fluids. When the concentration of cyanide in the suspensions is maintained reasonably constant through the use of such absorbing mixtures, respiration of *C. campylum* is found to behave like that of metazoan tissue cells. In Figure 2 are presented typical curves constructed from the data of a series of experiments performed under such conditions. Each point in the figure represents the average of the results obtained with two manometers. Again the concentrations of cyanide are approximate. The curves show

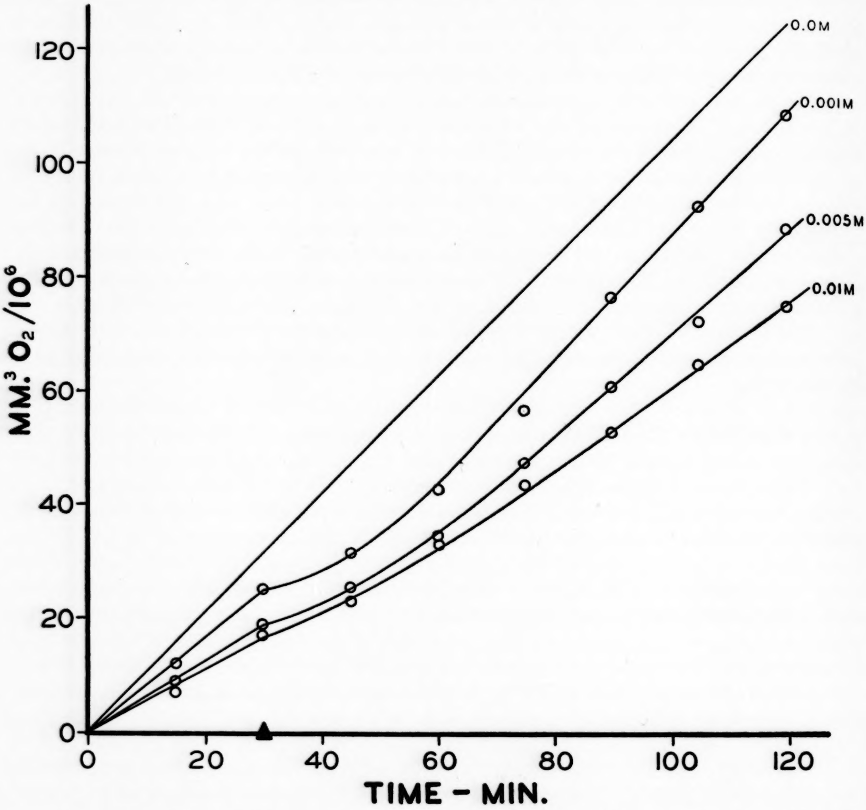

FIG. 1.—Effect of KCN on oxygen consumption of *C. campylum*. Respiratory CO_2 absorbed by KOH in inset. The figures at the right of the curves indicate the initial molar concentration of KCN.

that there is no recovery following initial inhibition of oxygen consumption. Respiration remains inhibited over a period of 90 minutes after the cyanide is tipped into the suspension. Readings were discontinued after this time. Attention is invited to the fact that, because of volatilization of HCN, the cyanide effect is present even before the KCN in

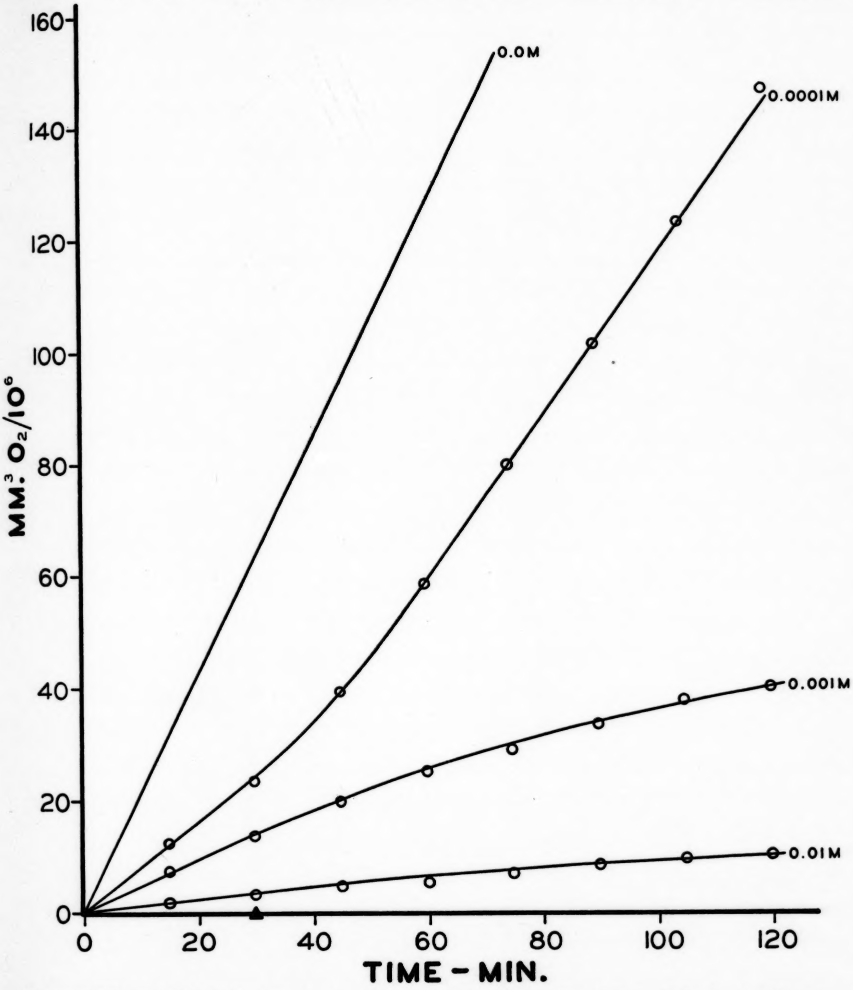

FIG. 2.—Effect of KCN on oxygen consumption of *C. campylum*. Respiratory CO_2 absorbed by means of the KCN-KOH mixtures recommended by Krebs (1935). The figures at the right, opposite the curves, refer to the initial molar concentration of KCN. The triangle on the time axis indicates the instant at which the KCN in the onset was tipped into the main chamber of the manometer vessel.

the onset chamber is tipped into the suspension. The break at 30 minutes is probably attributable in part to the shift in pH produced by the introduction of the KCN.

The extent to which respiration is inhibited is seen to depend on the concentration of cyanide. The effect is very nearly maximal with cyanide concentrations of the order of 0.001 M. The percentage inhibition with 0.001 M KCN is more than double that with 0.0001 M, while the increase with 0.01 M KCN over that with 0.001 M is very slight. The true percentage inhibition is found by comparing the rate of oxygen uptake in the experimental suspensions, 1 hour after tipping in the KCN, with the rate at corresponding pH in the absence of cyanide. The latter is obtained by interpolating along the curve fitted to the data of Table 1. The true percentage inhibition cannot be calculated by comparing the rate of oxygen consumption in the presence of a given concentration of KCN with the control for the experiment. Because of the pH change caused by the introduction of the KCN, the cells in the experimental suspensions are respiring at a different pH than the controls.

DISCUSSION

Pitts (1932) apparently was aware of the difficulty of measuring oxygen consumption in the presence of cyanide. However, in explaining the type of result which he obtained, he failed to recognize the true importance of the reversibility of the cyanide effect and the tendency for HCN to distil over into the KOH in the inset. Speaking of the recovery noted in his experiments, he states:

> This rise, following an initial inhibition, may be due in part to the volitalization of KCN, as shown by the appearance of the cyanide effect even when NaCN buffered at pH 7.4 is put in the side arm. It is extremely doubtful whether a major portion of the M/100 cyanide would have left the solution in the time involved. . . . .

From Pitts's description of the curve fitting his data there is little doubt that a major portion of the 0.01 M cyanide actually did leave the solution during the course of the experiments.

In testing the effect of cyanide on respiration, earlier investigators tried several methods of controlling exposure to the poison. Dixon and Elliott (1929) introduced the idea of neutralizing KCN in order to eliminate pH effects. In their experiments with medullated nerves Schmitt and Schmitt (cited by Robbie, Boell, and Bodine, 1938) replaced KOH as a CO_2-absorbing medium by solutions of cyanide salts sufficiently concentrated to take up the respiratory CO_2 and, because of the positive HCN-tension, also to prevent distillation of cyanide from the suspension medium. Van Heyningen (1935) was the first to employ KCN-KOH absorbing mixtures in respiration experiments. Robbie, Boell, and Bodine (1938) have checked the efficiency of these methods of control. They have shown that when no attempt is made to control exposure to cyanide, that is, when KOH is used to absorb respiratory CO_2, eggs of *Melanoplus* left in 0.0025 M KCN solutions recover their normal rate of respiration about as quickly as those removed and washed after a 3-hour exposure. Apparently, under these conditions HCN distils over into the KOH so rapidly that the cyanide concentration in the suspension medium is rather quickly reduced below the effective level for inhibition of respiration. When neutralized KCN is used, less than 30 per cent of the original amount remains in the suspension medium after 1 hour. Using a concentrated solution of KCN to absorb the respiratory CO_2, they have shown that in 3 hours the concentration of cyanide in the suspensions increases by 20 per cent of the original concentration, while in 6 hours it increases by 40 per cent. With the KCN-KOH absorbing mixtures recommended by Krebs (1935) the cyanide concentration in the suspension medium does not vary during a 6-hour period by more than 10 per cent of the original concentration.

A. Lwoff (1933) attempted to prevent loss of cyanide from his suspensions of trypanosomids by adding KCN to the KOH used in absorbing respiratory CO_2. The concentration of KCN in the absorbing mixture was adjusted so as to be equimolar with that in the experimental suspensions. M. Lwoff (1934) observed the same precaution in her experiments on *Glaucoma pyriformis*. In this connection it should be pointed out, however, that the fraction of cyanide in the absorbing mixture which would be effective in preventing loss from the suspension would be that which existed as free HCN, capable of participating in the diffusion equilibrium between both fluids and the gas space. In a strong KOH solution 0.001 M with respect to KCN, the concentration of free HCN would be very small indeed. In M. Lwoff's experiments the only effect of adding small quantities of KCN to the absorbing fluid was to prolong the recovery period. This was the result to be expected.

The causes of the difference between the results obtained by A. Lwoff (1933) for the animal flagellates and by M. Lwoff (1934) for *Glaucoma* cannot be stated at present with certainty. A possible explanation depends upon the fact that carbonic anhydrase was probably present in A. Lwoff's suspensions. A. Lwoff found that *Strigomonas fasciculata* and *Leptomonas ctenocephali* require small amounts of protohemin for growth in artificial media. The protohemin was supplied by adding fresh, defibrinated, hemolyzed dog's blood to the medium after sterilization. Carbonic anhydrase is typically present in mammalian blood (Brinkman, Margaria, Meldrum, and Roughton, 1932), from which special methods are required to separate it (Meldrum and Roughton, 1932). It seems probable, therefore, that in furnishing the necessary protohemin for the trypanosomids A. Lwoff also added carbonic anhydrase to the medium. This enzyme, which catalyzes the breakdown of H_2CO_3, is so powerful that one part in ten million is sufficient to double the rate of decomposition. By its presence, even in very small quantities, accumulation of carbonic acid in a suspension of respiring cells would tend to be prevented. Cyanide, if present, would tend to remain longer in the suspension, since the respiratory CO_2 would escape into the gas space and be absorbed by the alkali in the inset as rapidly as formed. While it is true that the enzyme is itself inhibited by cyanide, the minimum effective concentration (0.1 M, according to Meldrum and Roughton [1933]) of the poison required is much higher than for respiration. In A. Lwoff's experiments there was no recovery from cyanide poisoning because the concentration of KCN in the suspensions remained above the minimum effective level.

A similar explanation may be advanced to account for the difference between the percentage inhibition by cyanide of the respiration of ciliates and of mammalian tissue cells, where KOH alone has been used to absorb the respiratory CO_2. In preparing mammalian tissue slices for measurement of their respiration, some blood—and hence some carbonic anhydrase—must be included except when the slices are first washed. The variability of the "cyanostable" fraction of tissue-cell respiration reported by certain investigators (Dixon and Elliott, 1929) may depend upon the amount of carbonic anhydrase included in individual preparations. Using proper KCN-KOH absorbing mixtures, van Heyningen (1935) has shown that this fraction is universally small and quite constant.

There is no reason to suppose that carbonic anhydrase occurs in protozoa at all, and, indeed, in the case of *C. campylum* tests have failed to reveal its presence.[3] In the absence of carbonic anhydrase, carbonic acid would tend to accumulate in a suspension of protozoa respiring in a closed system (McBain [1912] and Thiel [1913] have shown that the breakdown of H_2CO_3 is ordinarily a relatively slow reaction). Distillation of HCN into the KOH would be accelerated by the accumulation of H_2CO_3 in the suspension medium. Consequently, respiration of the cells would tend to exhibit more rapid recovery than when carbonic anhydrase is present.

The sensitivity of respiration to poisons such as KCN, CO, and H_2S is affected by a number of factors, perhaps the most important of which is the extent of saturation of the respiratory system with carbohydrate (Warburg, 1927; Runnstrom, 1930; Keilin, 1932). A detailed discussion of these factors and their influence upon cyanide inhibition of respiration will be found in a recent paper by Commoner (1939). This investigator has shown that in concentrations of dextrose below a critical value the rate of respiration of yeast is not affected by KCN. The critical sugar concentration is lower for higher concentrations of the respiratory poison. Moreover, the maximum rate of respiration in the absence of cyanide varies as the critical dextrose concentration. Even endogenous respiration appears to be inhibited by 0.0002 M KCN. In the current research it was found that 0.0001 M KCN inhibits approximately 35 per cent of the oxygen consumption of *C. campylum* in a medium containing relatively little carbohydrate, while higher concentrations of the poison produce considerably greater inhibitions. In view of Commoner's (1939) findings these results suggest that the principal substrate oxidized by the protozoöns is carbohydrate and that the respiratory system of the cells is saturated with carbohydrate at the expense of decomposition products of the proteins in the medium.

The results of the present research, coupled with those of A. Lwoff (1933), indicate that a relative cyanostability characterizes the respiration neither of protozoa generally nor of the ciliates in particular. The protozoa react to cyanide in a manner similar to yeasts and tissue cells.

Since 88–95 per cent of the oxygen consumption of *Strigomonas oncopelti*, *S. fasciculata*, *Leptomonas ctenocephali*, and *C. campylum* is inhibited by 0.001 M KCN, it appears that 88–95 per cent of their respiration is dependent upon iron catalysis. A. Lwoff (1933) states that on spectroscopic examination suspensions of *S. oncopelti*, *S. fasciculata*, and *Glaucoma pyriformis* show a band at 555 mμ, which disappears when oxygen is bubbled through. In the case of *Glaucoma* the addition of $Na_2S_2O_4$ and pyridin causes the bands of the pyridin-hemochromogens of protohemin to appear. The prosthetic group of cytochrome is protohemin (Keilin), and that of cytochrome oxidase may also be (Warburg [1932] is of the opinion that this porphyrin is structurally intermediate between that of hemoglobin [protohemin] and that of leaves [phaeohemin]). From this it follows that both cytochrome and its oxidase may occur in protozoa. The effect of cyanide upon their rate of oxygen consumption suggests that the catalytic respiratory mechanism in protozoa, like that of most other aerobic cells, involves these iron compounds or closely similar ones.

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[3] The procedure described by Brinkman (1933) was employed in testing for carbonic anhydrase. Detection of the enzyme depends upon its effect on the velocity of the reaction between a 0.02 M solution of $NaHCO_3$, containing phenol red as an indicator, and a 0.006 M solution of CO_2 in water. The time required to attain the final yellow color of the indicator is taken as a measure of the reaction velocity. In the presence of carbonic anhydrase the velocity is greatly accelerated. In the current experiments fresh hemolyzed blood was employed as a standard containing carbonic anhydrase. The reaction was almost instantaneous. In the absence of any possible source of carbonic anhydrase the average reaction time was 63 seconds. With inactivated (heated for 2 minutes at 80° C.) samples of plasmolyzed ciliates, the average time was 67 seconds, while for untreated samples it was 69 seconds.

The author is unaware of any previous attempt to determine what relationship exists between oxygen consumption of protozoa and the pH of the medium. The majority of investigators heretofore either have made no effort to control the reaction of the medium in which the protozoa were suspended or have not considered this factor sufficiently important to warrant special attention. Indeed, the literature concerning the effect of H-ion concentration on oxygen consumption in general is limited, and, with the exception of the work of Tang (1936*a* and *b;* Tang and Mao-i Wu, 1938), those papers dealing with this subject which have appeared are controversial.

Warburg (1910) found that the oxygen uptake of sea-urchin eggs at pH 8.0 is nearly triple that at pH 6.0, while at pH 11.0 a further doubling occurs. The pH of the medium has been stated to be without significance as a chemical regulator of respiration of the rock barnacle, *Balanus crenatus* (Kreps, 1929) and of *Tubifex* (Dausend, 1931). In the former case, however, the measurements do not appear to have been carried out over a wide enough range to permit drawing conclusions, while in Dausend's (1931) experiments on *Tubifex* the actual magnitude of respiration was not determined, but only the frequency of movements of the hind end. Stahn (1928) found that respiratory activity of damsel-fly (*Aeschna grandis*) larvae increases as the H-ion concentration of the water from pH 7.0 to pH 6.5, then falls off with further increase in acidity. Similarly, Meyer (1935) reported a definite optimum for respiration of *Asterias* at pH 9.0. Although they do not give any supporting data, Shoup and Boykin (1931) state that for *Paramecium* "the region of change in the rate of oxygen consumption with change in pH is very narrow indeed."

In those instances in which the effect of H-ion concentration has been studied (Nomura, 1937; Tarantino, 1937), mammalian tissues have been found to exhibit a characteristic optimum pH of the medium for oxygen consumption. The results of the present investigation indicate that the behavior of *C. campylum* resembles that of tissue cells in this respect. The rate of oxygen uptake is maximum in the region of pH 5.5. It is regretted that for various reasons the measurements could not be carried out over a wider range of H-ion concentrations. It was found that the medium employed for cultivation of the ciliates would not support growth at pH 5.0 or lower, either with phosphate or with biphthalate buffers. Several other buffers were tried in an effort to extend the pH range. No growth occurred at all, however, in media buffered with citrate, borate, or paranitrophenol, regardless of the H-ion concentration.

According to Tang (1936*a*) the relationship between pH and the relative rate of oxygen uptake by *Saccharomyces Wanching* in acetate buffers between pH 4.2 and pH 5.8 can be represented by the empirical equation

$$\frac{d \log A}{d \text{ pH}} = K ,$$

where

$$A = \frac{\text{rate of } O_2\text{-uptake at pH} = x}{\text{rate of } O_2\text{-uptake at pH 5.8}} .$$

Integrating, we have

$$\log A = K \times \mathrm{pH}\ .$$

If $e^K = K'$, the equation may be written

$$\mathrm{pH} = \log A - \log K'\ .$$

This is identical with the logarithmic form of the equation developed by Michaelis and Davidsohn (1911) to describe the relation between H-ion concentration and the relative activity of yeast invertase on the acid side of the optimum pH.[4]

For reasons already stated, sufficient data were not obtained at pH below 5.5 in the present research to test the applicability of Tang's (1936*a*) equation. In phthalate buffers Tang found that respiration of *S. Wanching* is relatively insensitive to pH changes between 3.6 and 6.2 but falls off steeply to zero between pH 3.6 and pH 2.6. He believes that this difference from the behavior of *S. Wanching* in acetate buffers is due to the presence within the cells of substances which combine with phthalate and also with phosphate ions to form buffering systems capable of resisting considerable change in the H-ion concentration of the medium. If we accept this explanation, it would appear that the intracellular constituents which in *S. Wanching* enter into the formation of these buffer systems with phthalate and phosphate are not present in *C. campylum*. Curiously, in regard to these latter cells, acetate in sufficient concentration to be effective as a buffer seems to be distinctly toxic.

On the alkaline side of the optimum H-ion concentration Michaelis and Davidsohn (1911) found that the pH-activity relation for yeast invertase may be roughly described by an equation of the form

$$y = \frac{cH}{k_1 + cH} \qquad \text{or} \qquad \frac{1}{y} = 1 + \frac{k_1}{cH}\,,$$

---

[4] Michaelis' and Davidsohn's (1911) equation is:

$$y = \frac{k_2}{k_2 + cH}\,,$$

where

$$y = \frac{\text{rate of inversion at } cH = x}{\text{maximum rate of inversion}}\,,$$

and

$$k_2 = \frac{k_w}{k_b}\,,$$

$$k_b = \text{“basic” dissociation constant of enzyme}\ .$$

Writing the equation in a slightly different form, we have

$$\frac{1}{y} = 1 + \frac{cH}{k_2}\ .$$

The logarithmic form of this equation is

$$-\log y = \log cH - \log k_2\,,$$

hence

$$\mathrm{pH} = \log y - \log k_2\ .$$

where $k_1 = K_a$ = "acid" dissociation constant of enzyme, and

$$y = \frac{\text{rate of inversion at } cH = x}{\text{maximum inversion rate}} ,$$

$$= \text{"activity" of yeast invertase.}$$

The logarithmic form of the equation is

$$\log\left(\frac{1}{y} - 1\right) = \log k_1 - \log cH \; ;$$

hence

$$\text{pH} = \log\left(\frac{1}{y} - 1\right) - \log k_1 \; .$$

The applicability of this equation to the results of the current research has been tested by employing the average of the data obtained at a given pH-level in computing the activity coefficient y. The pertinent material is summarized in Table 2. It will be noted that the values obtained at different pH-levels for the negative logarithm of the constant k_1 (Col. 4 of the table) are in good agreement with the average, 7.65. The conclusion is permissible, therefore, that within the limits of experimental error the relationship between pH and the rate of oxygen consumption by *C. campylum* is given by the equation of Michaelis and Davidsohn (1911).

TABLE 2*

RELATION BETWEEN pH AND THE RATE OF OXYGEN CONSUMPTION OF *C. campylum*

pH	y	$\log\left(\frac{1}{y} - 1\right)$	$\text{pH} - \log\left(\frac{1}{y} - 1\right)$
5.56	0.994	$\bar{3}.778$	7.78
5.9	0.982	$\bar{2}.255$	7.65
6.44	0.936	$\bar{2}.833$	7.61
6.6	0.90	$\bar{1}.045$	7.55
7.0	0.822	$\bar{1}.337$	7.66
7.67	0.501	$\bar{1}.998$	7.67
Average	....	....	$7.65 = -\log k_1$

* Test of the fit of Michaelis and Davidsohn's (1911) equation

$$\text{pH} = \log\left(\frac{1}{y} - 1\right) - \log k_1$$

to the averaged data of Table 1.

The data relating oxygen consumption of *C. campylum* to the pH of the medium cannot be interpreted on the basis of what is now known regarding the metabolism of this species. The results suggest that some component of the oxidative mechanism in the cells is destroyed or irreversibly inactivated below pH 5.5, while on the alkaline side of the optimum pH some component is reversibly inactivated, or its action is inhibited, by the increasing hydroxyl-ion concentration.

It has been shown that hydrolyses, dehydrations, and some oxidations and reductions catalyzed by enzymes can also be catalyzed by hydrogen and hydroxyl ions. Enzymes are generally relatively stable in the region of their optimum pH but are universally destroyed by very strong acids or alkalis. Many are inactivated in fairly weak acid or alkaline solutions, and, in general, the pH-activity curves of enzymes have been found to resemble a typical dissociation-residue curve such as Michaelis and Davidsohn (1911) obtained for invertase. These properties of enzymes have been worked out with purified preparations from cell-free extracts. The properties of enzyme systems *in vivo* are largely unknown. The possibilities for investigation along these lines with simpler systems, where the factors involved are not too numerous to be handled conveniently, have been demonstrated by Wilkes and Palmer (1932), who showed that the curve for inversion-velocity by living yeast at different pH is identical, within the limits of experimental error, with the pH-activity curve of yeast invertase.

SUMMARY

Employing the technique of Krebs (1935) and van Heyningen (1935) for measuring the oxygen consumption in the presence of cyanide, we have shown respiration of *C. campylum* to be cyanide sensitive. The percentage inhibition of oxygen consumption increases rapidly with increasing cyanide concentration up to 0.001 M, after which the increase is slight. The average inhibition with 0.001 M KCN is 88 per cent. In a medium containing relatively little carbohydrate it was found that 0.0001 M KCN inhibits approximately 35 per cent of the oxygen consumption of *C. campylum*. In view of Commoner's (1939) findings with yeast cells, this result suggests that the principal substrate oxidized by the protozoa is carbohydrate and that the respiratory system of the cells is saturated with carbohydrate at the expense of products of the decomposition of proteins in the medium.

The absence of carbonic anhydrase in ciliate protozoa and the use of KOH solutions, or KCN-KOH mixtures containing insufficient cyanide, may account for the results of previous investigators, who have reported that oxygen consumption of these cells is relatively unaffected by cyanide.

The effect of cyanide on respiration of *C. campylum* suggests that the catalytic oxidative mechanism in ciliates, like that of most other aerobic cells, involves cytochrome and cytochrome-oxidase or some system closely resembling this one.

The relationship between oxygen consumption of *C. campylum* and the pH of the suspension was investigated. The maximum rate of oxygen uptake was observed at pH 5.5. On the alkaline side of this optimum the rate gradually fell off to about one-half the maximum at pH 7.65. Within this range of pH, the data were found to fit the equation of Michaelis and Davidsohn (1911):

$$\mathrm{pH} = \log\left(\frac{1}{y} - 1\right) - \log k_1 .$$

Below pH 5.5 the rate of oxygen consumption decreased sharply with increasing acidity. The data suggest that some component of the oxidative mechanism in these cells is destroyed or irreversibly inactivated below pH 5.5, while on the alkaline side of the opti-

mum some component is reversibly inactivated, or its action is inhibited by the increasing hydroxyl-ion concentration.

The medium employed for cultivation of the ciliates would not support growth at pH 5.0 or below, either with phosphate or with biphthalate buffers. No growth occurred in media buffered with acetate, citrate, borate, or p-nitrophenol, regardless of the pH.

LITERATURE CITED

BRINKMAN, R. 1933. The occurrence of carbonic anhydrase in lower marine animals. Jour. Physiol., **80**:171.

BRINKMAN, R.; MARGARIA, R.; MELDRUM, N. U.; and ROUGHTON, F. J. W. 1932. The CO_2 catalyst present in blood. Jour. Physiol. Proc., **75**:3.

CLARK, W. M. 1928. The determination of hydrogen ions. Baltimore, Md.: Williams & Wilkins Co.

COMMONER, B. 1939. The effect of cyanide on the respiration of baker's yeast in various concentrations of dextrose. Jour. Cell. and Comp. Physiol., **13**:121.

DAUSEND, K. 1931. Ueber die Atmung der Tubificiden. Zeitschr. f. vergl. Physiol., **14**:557.

DIXON, M., and ELLIOTT, K. A. C. 1929. The effect of cyanide on the respiration of animal tissues. Biochem. Jour., **23**:812.

FENYVESSY, B. VON, and REINER, L. 1924. Untersuchungen über den respiratorische Stoffwechsel der Trypanosomen. Zeitschr. f. Hyg., **102**:109.

HALL, R. H. 1938. The oxygen-consumption of *Colpidium campylum*. Biol. Bull. **75**:395.

HEYNINGEN, W. E. VAN. 1935. The inhibition of respiration by cyanide. Biochem. Jour., **29**:2036.

KEILIN, D. 1932. Cytochrome and intracellular respiratory enzymes. Ergebn. d. Enzymforsch., **2**:239.

KREBS, H. A. 1935. Metabolism of amino-acids. III. Deamination of amino-acids. Biochem. Jour., **29**:1620.

KREPS, E. 1929. Untersuchungen über den respiratorischen Gaswechsel bei *Balanus crenatus* bei verschiedenem Saltzgehalt des Aussenmilieus. II. Über den Einfluss von verschiedenem pH auf den O_2-Verbrauch und über die CO_2-Abgabe bei verschiedenem Saltzgehalt des Aussenmilieus. Arch. f. d. ges. Physiol., **222**:234.

LOEB, J., and WASTEN, H. YSE. 1913. Is narcosis due to asphyxiation? Jour. Biol. Chem., **14**:517.

LUND, E. J. 1918. Quantitative studies on intracellular respiration. II. The rate of oxidations in *Paramecium caudatum* and its independence of the toxic action of KCN. Amer. Jour. Physiol., **45**:365.

LWOFF, A. 1933. Die Bedeutung des Blutfarbstoffes für die parasitischen Flagellaten. Zentralbl. f. Bakt., **130**:498.

LWOFF, M. 1934. Sur la respiration du cilié *Glaucoma pyriformis*. Compt. rend. Soc. biol., **115**:237.

McBAIN, J. W. 1912. The use of phenolphthalein as an indicator: The slow rate of neutralization of carbonic acid. Jour. Chem. Soc., **101**:814.

MELDRUM, N. U., and ROUGHTON, F. J. W. 1932. Some properties of carbonic anhydrase, the CO_2 enzyme present in blood. Jour. Physiol. Proc., **75**:15.

———. 1933. The state of carbon dioxide in the blood. *Ibid.*, **80**:143.

MEYER, H. 1935. Die Atmung von *Asterias rubens* und ihre Abhängigkeit von verschiedenen Aussenfactoren. Zool. Jahrb., Abt. allg. Zool. u. Physiol., **55**:349.

MICHAELIS, L., and DAVIDSOHN, H. 1911. Die Wirkung der Wasserstoffionen auf das Invertin. Biochem. Zeitschr., **35**:386.

NOMURA, K. 1937. Influence of hydrogen-ion concentration on the tissue respiration of mouse and rabbit *in vitro*, attention being paid to the buffer potency of the tissue. Tohuku Jour. Exper. Med., **31**:19.

PITTS, R. F. 1932. Effect of cyanide on respiration of the protozoan *Colpidium campylum*. Proc. Soc. Exper. Biol., **29**:542.

ROBBIE, W. A.; BOELL, E. J.; and BODINE, J. H. 1938. A study of the mechanism of cyanide inhibition. I. Effect of concentration on the egg of *Melanoplus differentialis*. Physiol. Zoöl., **11**:54.

RUNNSTROM, J. 1930. Atmungsmechanismus und Entwicklungserregung bei Seeigelei. Protoplasma, **10**:106.

SHOUP, C. S., and BOYKIN, J. T. 1931. The insensitivity of *Paramecium* to cyanide and effects of iron on respiration. Jour. Gen. Physiol., **15**:107.

SØRENSEN, S. P. L. 1909. Ueber die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen. Biochem. Zeitschr., **21**:131.

STAHN, I. 1928. Über die Atmungsregulation, besonders die Kohlensäureregulation, bei *Dixippus morosus* und *Aeschna grandis:* Ein Beitrag zur Atmung der Insekten. Zool. Jahrb., Abt. allg. Zool. u. Physiol., **46**:1.

TANG, P. S. 1936*a*. Studies on the kinetics of cell respiration. I. The rate of oxygen consumption of *Saccharomyces Wanching* as a function of pH. Jour. Cell. and Comp. Physiol., **7**:475.

———. 1936*b*. Studies on the kinetics of cell respiration. VI. Respiration of *Chlorella pyrenoidosa* in the presence of lactates. *Ibid.*, **10**:499.

TANG, P. S., and WU, MAO-I. 1938. Studies on the kinetics of cell respiration. VII. Respiration of *Saccharomyces Wanching* in acetate, lactate, and pyruvate buffer solutions. Jour. Cell. and Comp. Physiol., **11**:495.

TARANTINO, C. 1937. La Concentrazione idrogenionica del mezzo in funzione del consumo di O_2 del tissuti. Riv. biol., **23**:281.

THIEL, A. 1913. Ueber die langsame Neutralisation der Kohlensäure. Ber. deutsch. chem. Gesellsch., **46**:241.

WARBURG, O. 1910. Ueber die Oxydationen in lebenden Zellen nach Versuchen am Seeigelei. Zeitschr. f. physiol. Chem., **66**:305.

———. 1914. Beiträge zur Physiologie der Zelle, insbesondere über die Oxydationsgeschwindigkeit in Zellen. Ergebn. d. Physiol., **14**:253.

———. 1927. Wirkung von Kohlenoxyd und Stickoxyd auf Atmung und Gärung. Biochem. Zeitschr., **189**:354.

———. 1932. Das sauerstoffübertragende Ferment der Atmung. Zeitschr. angew. Chem., **45**:1.

WILKES, B. G., and PALMER, E. T. 1932. Similarity of the kinetics of invertase action *in vivo* and *in vitro*. II. Jour. Gen. Physiol., **16**:233.

GROWTH STUDIES ON CILIATES. V. THE ACCELERATION AND INHIBITION OF CILIATE GROWTH IN BIOLOGICALLY CONDITIONED MEDIUM[1]

(Six figures)

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EVER since the observations of Robertson (1922–27) on the growth rates of certain ciliates and their variations in response to what he called "allelocatalytic effects," there have appeared from time to time numerous papers dealing with this subject. Some of the early workers (Cutler and Crump, 1923*a*, 1923*b*, 1924, 1925) dealt directly with the problem and attempted to repeat Robertson's experiments, while others reported either support or contradiction of his hypothesis on the basis of dissimilar experiments. The general question of "allelocatalysis" has been reviewed too frequently in the past to warrant any lengthy discussion here other than to say that it is far from settled at present. Excellent discussions of the status of the problem are to be found in the works of Allee (1931, 1934), Mast and Pace (1938), and Park (1939).

It is only within the past few years that the literature has contained accounts of experiments, carried on under controlled conditions, which would throw any light upon the question as to whether the acceleration of growth or the depression of growth, often noted, could be due to the metabolic activities of the protozoa or to the associated microorganisms. With the use of bacteria-free saprozoic protozoa becoming more and more extensive one should expect this question to be revived. To date only a few papers have been published in which the problem mentioned above has been attacked under conditions permitting control of the experimental cultures by the elimination of bacteria.

Phelps (1935) in his excellent study of *Tetrahymena* (*Glaucoma*) regarding the relationship between the size of the inoculum and the growth rate found no evidence of the "Robertson effect." The growth rate and the maximum yield were the same whether he started his culture with 0.014 ciliates per ml. or 1,600 ciliates per ml. On the other hand, Mast and Pace (1938), using the flagellate *Chilomonas*, reported a significant acceleration of growth rate when many chilomonads were inoculated into a small volume of culture fluid as compared to the growth rate of single flagellate inocula. They further report that culture medium in which chilomonads had been growing for a short time possessed a substance, elaborated by the flagellates, which accelerated the growth of freshly inoculated chilomonads. This substance, according to Mast and Pace, inhibits growth, however, if its concentration becomes too great, as in the culture fluid in which a population had multiplied and died. They later (1939) reported that the substance has a molecular size not greater than 6 mμ because of its ability to diffuse through cellophane.

With these and other observations of protozoölogists, together with the many interesting reports of like phenomena among the bacteria, yeasts, and molds and even a number of the higher invertebrates as a basis, it was thought worth while to examine the problem by employing slightly different techniques. The use of bacteria-free ciliates affords one the opportunity to study cell-free conditioned medium by the simple expedient of centrifugation—a technique difficult when bacteria are being studied, especially when large quantities of medium are needed. It is, therefore, the purpose of this paper to report the results of a study of the effects of biologically conditioned medium upon the growth rate of bacteria-free ciliates of the genus *Tetrahymena* (Furgason, 1940).

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MATERIAL AND METHODS

The strain of ciliates used in this study was originally obtained from Dr. Robert H. Hall and is the one used by him for his study on oxygen consumption (Hall, 1938). It has been known in the literature as *Colpidium campylum* and was so designated in earlier reports from this laboratory (Kidder, 1939*a*, 1939*b;* Kidder and Stuart, 1939; Kidder, Lilly, and Claff, 1940) but following the investigation of Furgason (1940) it will be referred to as *Tetrahymena geleii* (Hetherington strain).

The stock ciliates were grown in 2 per cent Difco proteose-peptone–1 per cent Difco dextrose broth made up with Pyrex distilled water. This same medium was used for the bulk of the experiments here reported, although a few experiments using 1 per cent Difco yeast extract demonstrated that the general effects were the same for both.

In order to follow the growth of the populations it is necessary to make frequent counts. This involves withdrawal of volumes of medium from the growing culture; and when tubes are used the reduction soon becomes a very important factor in future growth. In the early part of the work test tubes were used, two hundred tubes of each series were inoculated, and ten tubes were sacrificed for counting purposes at each time interval; the average count was used in the records. This method was soon discarded, however, as expensive of materials and time. The bulk of the experiments were carried on in specially designed flasks.

The design of the culture flask used will be given here in detail, as it is believed that it should prove useful to future investigators of protozoan, bacterial, and yeast growth. To an ordinary Erlenmeyer flask (either 250-ml. or 1,000-ml. capacity) a side arm is fused. This side arm is of the same diameter as the mouth of the flask and is set at an angle to it. The purpose of the side arm is for inoculation in case the upper opening is used for an aeration apparatus. The aeration apparatus is placed in the flask before sterilization and need not then be removed. Near the bottom of the flask is fused a Pyrex port, shaped like the neck of a vaccine ampule, which will accommodate a rubber vaccine cap. This port is for the aseptic removal of culture medium for the purpose of counting. Before sterilization the whole port with rubber cap in place is wrapped in cotton (Fig. 1, *A*). When counts are to be made, a piece of sterile cotton is saturated with 70 per cent alcohol and substituted for the dry cotton. The flask is then agitated to insure even distribution of protozoa. With a No. 18 (2-inch) hypodermic needle, previously sterilized in a cotton-plugged serological tube (Fig. 1, *B*) and attached to a syringe (Fig. 1, *C*) before removal from the tube, the vaccine tip is punctured, the needle inserted into the body of the culture, 1 ml. or more is withdrawn, and the cotton is replaced. This sample is then delivered into a vial or tube.

By this means small volumes may be withdrawn from a single flask at frequent intervals; and, when the proper technique is mastered, the danger of contaminating the flask is practically eliminated. A single tip may be entered as many as fifty times without leaking. When 100 ml. or more of culture medium is used in a flask, the reduction of volume, even over a long period of time, is not an important factor. These flasks have proved invaluable to us, not only in the present study, but in other investigations on protozoan growth as well.

In order to test the possible presence of growth substances elaborated into the medium by the ciliates the following technique was used. Medium was prepared, and one flask was filled and set aside to act as the control on the experimental flasks. The rest of the medium was distributed into balanced, heavy-duty centrifuge tubes of 40-ml. capacity. The flask and the tubes were plugged and autoclaved. After cooling, the tubes were inoculated with stock ciliates, each tube receiving approximately the same number of ciliates (usually 100 per ml.). Growth was allowed to take place for varying periods of time, according to the experiment. This was the period of conditioning. The tubes were then all immersed in a water bath at 40° C. for 10 minutes to kill the ciliates or render them inactive. Half of the tubes were centrifuged at 3,900 r.p.m. for 6 minutes, which was sufficient to throw the ciliates to the bottom. The clear supernatant fluid of each tube was then withdrawn with a sterile 50-ml. volumetric pipette and delivered into a sterile culture flask. A fresh pipette was used for each tube, and the volume in the flask made up to 100 ml. when 250-ml. flasks were used, or to 500 ml. when liter flasks were employed. Within any single experiment all flasks were of the same size and contained the same volumes. Centrifugation (with cotton-plugged tubes) and subsequent transfers were carried out aseptically. This supernatant fluid from the ciliate culture was designated as "centrifuged-conditioned medium."

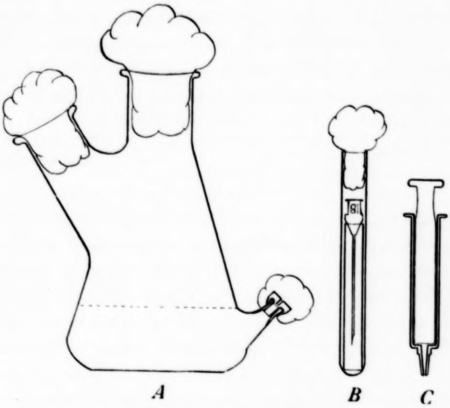

FIG. 1.—Diagram of culture flask, *A*, showing the position of the port with vaccine tip in place. The dotted line represents the height of the culture fluid. *B* shows the method used in the sterilization of hypodermic needles, which are placed on syringe *C* for use.

The fluid from the other half of the tubes was passed through sterile bacteriological filters (usually Seitz, but occasionally Berkfeld), and the sterile, ciliate-free filtrate was then transferred aseptically to flasks for future use. This filtrate was designated as "filtered-conditioned medium." Frequently, when the Seitz filter was to be employed, one operation was eliminated by sterilizing the filter in the culture flask which was to be used and then filtering directly into the flask by means of positive pressure. The flasks are too thin to allow for vacuum filtering, although the side arm can be used for suction attachment. When the requisite amount of filtrate had collected in the flask the filter was removed and a sterile cotton plug substituted.

After the two types of conditioned media had been prepared, all three flasks (unconditioned control, centrifuged-conditioned, and filtered-conditioned) were inoculated with equal volumes of the same stock ciliate culture, and the size of these initial inocula was determined for each flask by drawing samples and counting the ciliates present. Thereafter counts were made at frequent intervals (2 or 3-hour intervals during the early growth phases), and growth curves constructed from the data.

Direct counts were made of living ciliates in order that a comparison might be made between the data from these experiments and the somewhat similar data from bacterial studies, because most of the bacterial studies had utilized viable counts. Five to ten samples were always counted, and the results averaged. When dilutions were necessary as little time as possible was taken from the time the sample was drawn until the count was made, as it was thought possible that fresh broth (the diluent) might prove harmful to the ciliates from conditioned broth and tend to render the counts low.

All experimental flasks were incubated at 26° C. At the end of each experiment sterility tests both in liquid and on solid media were made, and the tests were kept two weeks for the detection of possible slowly growing contaminants. All data recorded in this report are from bacteriologically sterile cultures.

Variations from the above procedure, including the age of the inocula, will be noted and described later in the discussion.

THE NORMAL GROWTH CURVE

Several experiments were performed to determine the normal growth characteristics of *Tetrahymena* in the proteose-peptone–dextrose medium which was to be used for the conditioning investigations. The shape and slope of the curve were found to be comparable in every respect to those given by Phelps (1935) for his strain of *Tetrahymena* (*Glaucoma*). Ciliates inoculated from the logarithmic phase into fresh medium continued to reproduce at the same rate as they were doing in the previous culture. In other words, inoculum from the logarithmic phase of growth exhibited no lag phase (see control, Figs. 2, 3, 4). The numbers of ciliates present per unit volume (ml.) when plotted on a semilog scale against time give a straight line, which, when the initial inoculum is approximately 100 cells per ml., continues for from 36 to 40 hours. The slowing of the division rate is then quite abrupt, so that the curve invariably bends over sharply during the negative acceleration phase, and the stationary phase ensues. This is not at once a real stationary phase, however, as multiplication always exceeds death for many hours after the reduced rate of reproduction begins. Cultures followed for many days show the characteristic sharp logarithmic growth phase and the short negative acceleration period, followed by a slowly rising population which does not decline appreciably for weeks. For the purposes of this investigation the first part of the growth curve was selected for study, and none of the comparative experiments were continued more than 132 hours, the majority being terminated at 96 hours. Detailed data are limited to the first 72 hours in this report.

These ciliates exhibit the same general characteristics as do bacteria in that a lag phase is evidenced if the inoculum is taken from old cultures. The length of the lag phase is roughly and within limits proportional to the length of time the ciliates have spent in their old environment after the logarithmic phase of growth is passed. Ciliates taken from a 48-hour culture show very little lag, those from a 60-hour culture more, while those taken from a 96-hour culture may show very little if any multiplication for from 4 to 6 hours (see control, Fig. 5). However, the number of ciliates per ml. at the end of logarithmic growth and the maximum yield are independent of the age of the inoculum. It was decided, for the purposes of this study, that comparisons of conditioned media with control media could be made best if complications of a lag phase were omitted. Accordingly, the standard age of the inoculum for all but one set of experiments was 24 hours, or well within the logarithmic phase.

The normal growth curve (see control, Figs. 2, 3, and 4) for *Tetrahymena* (Hetherington strain) when grown in 100-ml. volumes of the proteose-peptone–dextrose medium is remarkably constant and can be reproduced at will.

TABLE 1

EFFECT OF CONDITIONED MEDIUM

MEDIUM	EXPT. No.	HOURS												
		0	2	4	6	8	10	12	21	24	30	36	48	72
Control.......	1	100	155	200	310	480	660	980	5,900	9,600	28,500	78,000	125,000	162,000
	2	110	170	200	350	470	680	1,100	6,200	11,000	26,000	68,500	94,000	151,000
	3	100	150	210	300	480	600	960	5,700	9,800	30,000	82,500	135,000	177,000
	4	125	165	220	370	500	700	1,150	6,100	12,500	28,500	76,000	146,000	168,000
	5	100	175	190	320	490	690	1,200	5,900	13,000	27,000	91,000	130,000	173,000
	6	110	170	180	325	480	660	1,000	5,800	9,500	29,000	60,500	142,000	149,000
Av.......	.....	107	164	200	328	480	665	1,065	5,933	12,580	28,180	76,083	128,666	163,333
Conditioned (60-hour centrifugate)...	1	100	165	300	640	1,150	2,000	3,150	17,200	19,200	27,300	31,500	38,500	38,500
	2	135	190	310	750	1,600	2,600	3,950	20,500	26,100	34,200	38,000	38,000	38,500
	3	98	150	350	580	1,200	1,750	2,600	11,000	13,600	21,500	27,000	31,000	30,000
	4	100	155	280	600	990	2,150	3,900	12,000	19,000	27,500	32,500	34,500	32,500
	5	90	150	290	660	1,380	2,300	3,750	15,500	17,500	25,000	29,000	39,000	31,000
	6	100	165	300	560	1,420	2,200	3,840	19,500	21,400	19,000	26,500	36,000	35,000
Av.......	.....	104	162	310	631	1,290	2,166	3,531	15,950	19,466	25,750	30,750	36,166	34,250
Conditioned (60-hour filtrate)......	1	120	100	108	115	160	230	330	1,800	3,250	7,800	12,500	20,000	40,000
	2	100	110	115	120	170	260	360	2,300	3,700	8,650	14,200	26,000	36,000
	3	100	100	110	115	160	210	320	1,600	3,350	7,250	12,100	19,500	42,000
	4	85	80	90	100	120	200	290	1,200	2,600	6,550	9,900	19,500	41,500
	5	100	100	107	112	190	250	350	2,000	3,150	8,100	13,100	18,000	38,000
	6	120	110	117	121	180	240	310	2,000	3,450	7,750	12,000	16,500	43,500
Av.......	.....	104	100	107	117	163	231	326	1,816	3,250	7,683	12,300	19,916	40,166

EFFECTS OF CONDITIONED MEDIUM

When a population of ciliates has grown in a medium, they produce a change which affects the growth of a subsequent population of ciliates inoculated into the same medium. The effects are noted in (1) acceleration of division rate or (2) inhibition of growth for a period of time, depending upon the manner in which the previous population has been removed from the medium. Removal by centrifugation leaves a medium which accelerates growth; removal by filtration gives a medium which temporarily inhibits growth. The intensity of acceleration increases with the time of conditioning up to 60 hours, under the conditions of these experiments, and then decreases with further conditioning. Inhibition by the filtrate increases, within limits, directly in proportion to the time of conditioning.

MEDIUM CONDITIONED 60 HOURS

This series was repeated twelve times. The outcome of all twelve of the experiments was practically identical. Data from six experiments are presented in Table 1, and the averages are plotted in Figure 2. The six experiments presented were selected from the whole solely on the basis of time counts, all these six being counted at exactly the same time intervals. This selection was done to facilitate presentation of tabular data. Individual curves drawn from the other six are identical with the ones presented here but have been omitted for lack of space.

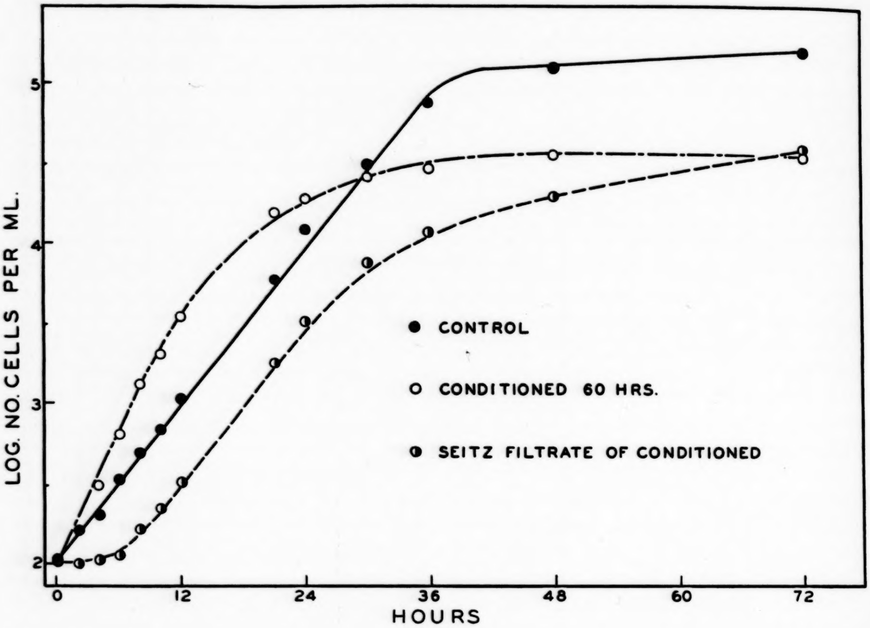

FIG. 2.—Diagrammatic representation of the data given in Table 1

It will be seen at a glance that, whereas the control cultures exhibited perfect logarithmic growth during the first 36 hours, the centrifuged-conditioned cultures show very significant acceleration over the control. This rapid reproductive rate always begins to decline early, however, and the curve flattens out very much sooner than in the control. The maximum yield is never as high as in the control. The accelerating effect is therefore evidenced only during the early growth phases, the medium proving to be below optimum for maximum population yield.

Conditioned medium from which the ciliates have been removed by either a Seitz or a Berkfeld filter is inhibitory during the early growth phases. The counts and the curves show that a pronounced lag phase appears, but the yield at 72 hours equals or slightly exceeds that of the centrifuged-conditioned medium. It was thought at first that this

TABLE 2

EFFECT OF CONDITIONED MEDIUM

MEDIUM	EXPT. NO.	HOURS											
		0	2	4	6	10	12	15	24	28	36	48	72
Control	1	130	160	260	370	730	1,500	2,000	9,000	19,000	46,000	90,000	120,000
	2	110	140	200	310	500	800	1,200	7,100	14,500	46,500	100,000	110,500
	3	150	180	300	420	680	1,200	1,700	8,500	17,000	42,500	60,000	75,000
Av.	.....	130	160	253	366	636	1,166	1,633	8,200	16,830	45,000	83,333	101,806
Conditioned (4-week centrifugate)	1	100	170	360	570	1,140	1,500	2,200	8,500	11,600	16,400	15,500	26,000
	2	50	85	160	310	850	1,100	1,600	6,000	10,500	15,200	25,500	50,000
	3	150	240	450	810	1,600	2,400	3,950	10,500	12,500	17,500	36,500	32,000
Av.	.....	100	165	323	563	1,196	1,666	2,583	8,333	11,533	16,360	25,500	36,000
Conditioned (4-week filtrate)	1	90	80	85	100	160	200	360	1,750	2,800	6,250	13,500	39,000
	2	120	100	100	120	180	230	330	2,000	4,050	7,000	16,000	41,000
	3	110	95	100	110	170	240	290	1,200	1,950	5,500	9,000	40,000
Av.	.....	106	91	95	110	170	223	326	1,650	2,933	6,250	12,833	40,000

TABLE 3

EFFECT OF CONDITIONED MEDIUM

MEDIUM	EXPT. NO.	HOURS									
		0	2	6	8	12	20	30	48	60	72
Control	1	100	130	240	410	700	2,500	16,300	70,000	71,000	78,000
	2	80	100	210	370	650	1,900	16,000	72,000	80,000	100,000
	3	160	210	400	620	1,000	2,700	18,500	68,000	69,000	80,000
Av.	.....	113	146	283	466	786	2,366	16,933	70,000	73,333	86,000
Conditioned (8-week centrifugate)	1	100	110	200	250	410	980	2,200	16,000	25,500	20,000
	2	90	100	160	210	300	760	1,800	14,000	26,000	24,000
	3	110	130	230	300	460	900	2,100	17,000	21,000	19,000
Av.	.....	100	113	196	253	390	880	2,033	15,666	24,133	21,000
Conditioned (8-week filtrate)	1	100	90	100	100	120	350	1,550	13,000	20,000	29,000
	2	100	80	90	80	110	320	1,700	10,000	26,000	36,000
	3	110	100	110	110	140	400	2,100	13,500	16,000	16,000
Av.	.....	103	90	100	96	123	356	1,783	12,166	20,666	27,000

inhibition might be due to materials added to the medium from the filter. Accordingly, fresh medium was filtered and inoculated, along with normal controls. This filtered fresh medium exhibited no inhibitory effect. The inhibition of the conditioned filtrate, then, appears to be due to the conditioning action of the ciliates. It is possible, therefore, to demonstrate both "beneficial" and "detrimental" results of the growth products of a ciliate population.

It should be noted that in these twelve experiments the growth rates for the three types of experimental media never overlapped during the first 24 hours. This means that the averages give a true representation of the actual numbers of ciliates in any one experiment at any given time.

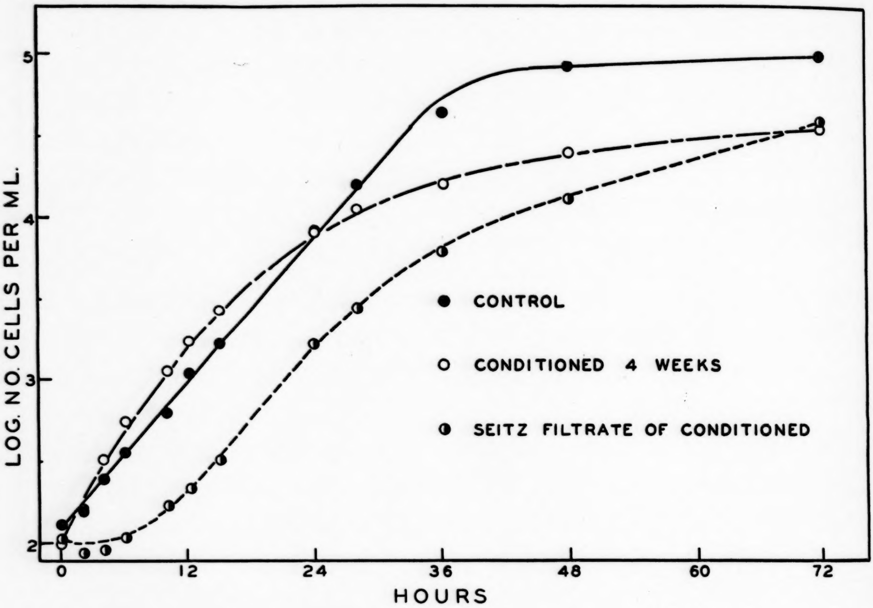

FIG. 3.—Diagrammatic representation of the data given in Table 2

MEDIUM CONDITIONED 4 AND 8 WEEKS

Inasmuch as cultures of *Tetrahymena* will remain at a high population level for many weeks, it was thought advisable to test the effect of conditioning over a long period of time. Accordingly, two series of experiments were planned, the first testing the effect of medium conditioned 4 weeks, and the second of medium conditioned 8 weeks. For these experiments liter flasks were used for the conditioning cultures. A number of flasks were set up and inoculated at varying intervals; and when the desired age was reached half the contents of a flask was transferred aseptically to centrifuge tubes, and the other half was filtered. Eight experiments were completed with the 4-week conditioned medium, and five with the 8-week conditioned medium. The results of three experiments each are given in Tables 2 and 3, and the averages are plotted graphically in Figures 3 and 4.

In medium conditioned 4 weeks and having the previous population removed by centrifugation, acceleration of growth occurred as it did in the 60-hour experiments. The acceleration was less marked, however, and the rate of reproduction decreased much sooner. The maximum yield was approximately the same as in the previous case.

The filtrate of the 4-week conditioned medium again produced inhibition, and the lag phase was somewhat longer. Again the maximum yield was about the same as that of the centrifugate.

It is apparent that conditioning up to 4 weeks renders the medium less suitable for a subsequent population than conditioning for a few (60) hours. The accelerating effect, though still significant, is diminished, while the inhibitory effect of the filtrate is more pronounced.

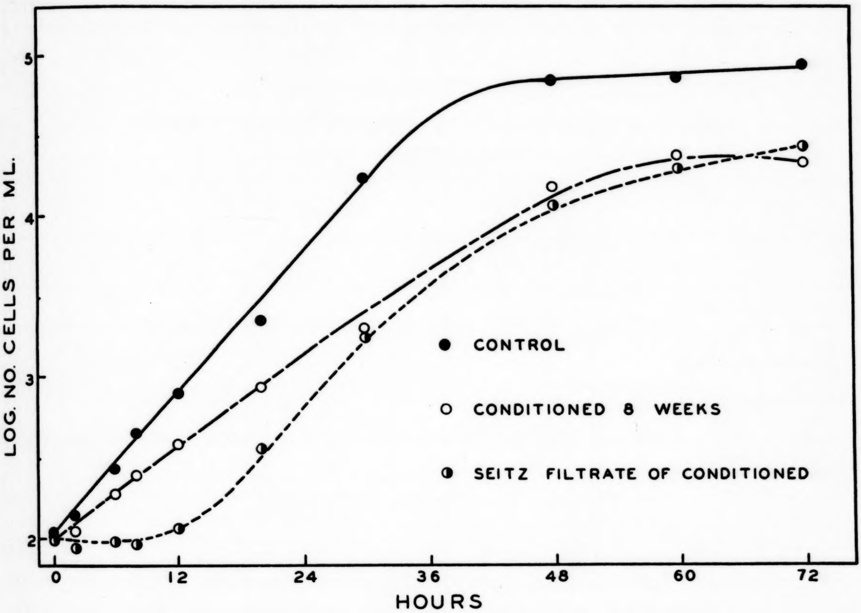

FIG. 4.—Diagrammatic representation of the data given in Table 3

After conditioning for 8 weeks the centrifugate no longer exhibited the accelerating effect as compared to the fresh medium control. The division rate was at all times less than the control, but, in comparison to the filtrate, no apparent lag phase took place.

The filtrate of the medium conditioned for 8 weeks bore the same general relation to the centrifugate during the first few hours of growth, as has been previously noted; but curiously enough the ciliates divided at a higher rate than those in the centrifugate, once the lag phase had been passed.

ADSORPTION EXPERIMENTS

Both the asbestos pads of the Seitz filter and the candle of the Berkfeld filter possess an electronegative charge. It was thought that there was a possibility that the accelerative substance, if such existed, was being adsorbed during filtration and that therefore any electronegative substance would produce the same effect even without the mechanics of filtration. Accordingly, experiments were conducted wherein animal charcoal (Norit) was used as the adsorbent. The Norit was placed in dry, 40-ml. centrifuge tubes and sterilized for 2 hours at 145° C. in a hot-air oven. After the Norit had cooled, the centrifugate from a 60-hour culture was added aseptically and mixed by shaking. The Norit was then centrifuged down, and the supernatant fluid was placed in culture flasks, 100 ml. per flask. At the same time three other types of flasks were prepared—one substituting washed plaster of Paris (electropositive) for the Norit, one with centrifuged 60-hour conditioned medium, and one with unconditioned medium. The last two served as controls. The results of these experiments are given in Table 4 and shown graphically in Figure 5.

TABLE 4

EFFECT OF ADSORPTION ON CENTRIFUGED CONDITIONED MEDIUM

(Average of two experiments)

Medium	Hours											
	0	2	4	6	8	12	16	24	30	36	48	72
Control I (untreated medium)	240	350	510	800	1,050	1,900	4,800	20,000	63,000	155,000	240,000	260,000
Control II (conditioned 60-hour centrifugate)	160	405	660	1,100	2,050	4,600	12,400	38,000	61,000	59,000	61,000	55,000
Conditioned 60 hours (adsorbed with Norit)	180	220	290	350	690	1,300	2,500	12,400	31,000	47,000	56,000	58,500
Conditioned 60 hours (adsorbed with plaster of Paris)	170	160	175	180	210	400	830	3,900	12,900	31,000	50,000	58,000

The Norit-treated medium exhibited the same degree of inhibition of growth as did the filtrate in the previous 60-hour experiment. The lag phase was of about the same duration, and the general shape and height of the growth curve was the same.

The medium treated with plaster of Paris yielded somewhat surprising results. Not only was there inhibition of growth, but the lag phase was extremely long, and it was not until after 12 hours that the division rate was as high as in the Norit-treated medium.

These experiments were repeated with the same results. The inhibiting effect could not be due entirely to the adsorption of some accelerating substance unless the substance is capable of shifting the sign of its electrical charge, because both electronegative and electropositive materials acted in the same manner. Separate experiments, wherein plain, unconditioned medium was treated as above with both Norit and plaster of Paris, yielded growth curves similar to the untreated controls. No materials essential for maximum growth under these conditions appear to be removed from normal medium, nor do detrimental substances appear to be added.

TEMPERATURE AND TIME

The effects of boiling temperatures on the 60-hour conditioned medium after the cells had been removed was tested. Both the accelerative effect of a centrifugate and the inhibiting effect of a filtrate are completely destroyed at 100° C. (Table 5). Boiled centrifugate and filtrate lose their conditioned effect and produce curves comparable to the normal control curves but with a lower maximum yield.

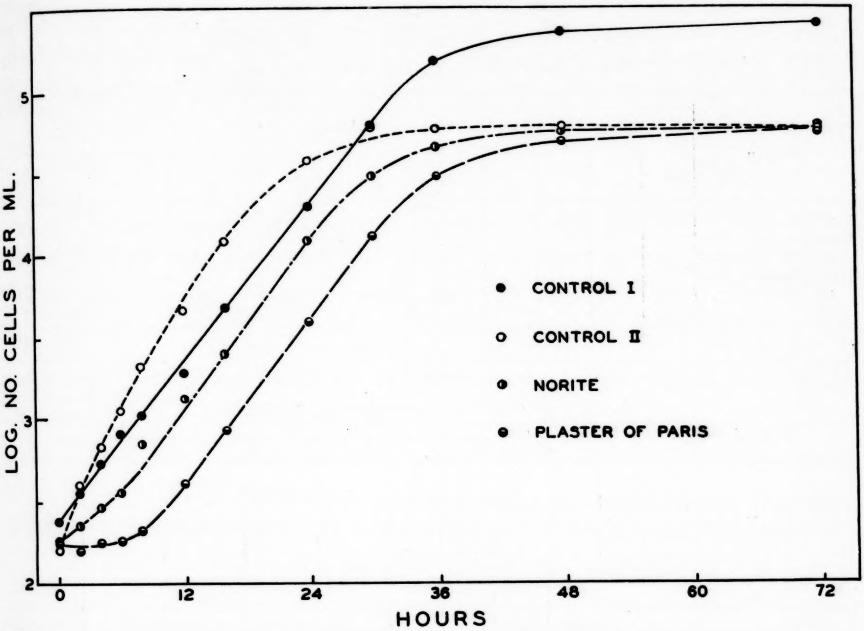

FIG. 5.—Diagrammatic representation of the data given in Table 4

If 60-hour conditioned medium is handled as described in the first set of experiments (Table 1) but allowed to stand uninoculated for 72 hours after the previous population has been removed, both the accelerating effect of the centrifugate and the inhibiting effect of the filtrate diminish but do not completely disappear (Table 6). Conditioning effects do disappear after 10 days, however.

ACCELERATION AND THE LAG PHASE

As was previously stated, no lag phase appears if ciliates from the logarithmic phase of growth are inoculated into untreated proteose-peptone–dextrose medium. When ciliates from an old culture are used for inoculation, however, a lag phase[2] appears. What effect will the accelerating substance of centrifuged conditioned medium have on old ciliates immediately after inoculation? To answer this question a centrifugate of medium conditioned 60 hours, together with a control of untreated medium, was inoculated with ciliates from a 7-day-old culture. This experiment was repeated three times, and the data from the four experiments are given in Table 7 and shown graphically in Figure 6.

The general relationship of 7-day inoculum in the centrifuged, 60-hour medium to 7-day inoculum in the control is the same as when young ciliates (taken from the logarithmic phase) are used. The ciliates exhibit only a slight indication of a lag phase in the former medium and an appreciable lag in the control. This is a very interesting point in that it has long been accepted that, in bacterial and yeast growth, a lag phase is the inevitable outcome of old inocula.

TABLE 5

EFFECT OF HEATING OF CONDITIONED MEDIUM

(100° C. for 5 minutes; average of four experiments)

MEDIUM	HOURS									
	0	2	6	10	12	24	36	48	60	72
Control	90	124	250	520	790	7,700	66,000	102,000	110,000	125,000
Conditioned (60-hr. centrifugate)	75	90	185	410	600	5,200	15,200	31,000	46,000	70,000
Conditioned (60-hr. filtrate)	103	155	330	720	1,050	8,000	17,000	25,000	39,000	51,000

TABLE 6

EFFECT OF AGING OF CONDITIONED MEDIUM

(Inoculated 72 hours after removal of previous population; average of two experiments)

MEDIUM	HOURS								
	0	4	6	10	12	24	36	60	72
Control	120	200	330	610	900	8,600	31,000	78,000	100,000
Conditioned (60-hr. centrifugate)	100	260	490	920	1,400	8,800	16,000	29,000	36,000
Conditioned (60-hr. filtrate)	100	105	125	300	410	3,500	14,000	31,000	46,000

If one thinks of the lag phase as being a period of adjustment or acclimatization to a new environment, then the above result might be expected. The medium which has been conditioned for 60 hours is more nearly like the medium from which the inoculum was taken than is fresh broth. At any rate this seems to indicate that the cause for a lag phase, which may be due to the previous environment of the inoculated organisms, may be modified considerably by the choice of media one may use.

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[2] The term "lag phase" as used in this discussion is the period elapsing between the inoculation and the beginning of logarithmic growth. No attempt has been made—and the data does not warrant such an attempt—to break this phase up into the "initial stationary phase" and the "lag phase" of Buchanan (1918).

DISCUSSION

The data which have been accumulated with the use of the techniques described above allow one to put forth some tentative interpretations regarding their meaning. It is possible to obtain duplications of the results of any of the sets of experiments under these conditions, and one might be tempted to generalize as to their meaning. As a matter of fact, the general phenomena reported here for *T. geleii*(Hetherington strain) apply equally well to at least two other strains of the same species (Elliott strain and Phelps strain), as I have previously reported (Kidder, 1939*b*). Whether these results will be obtained when still other species and genera are employed is, of course, impossible to say.

TABLE 7

EFFECT OF CONDITIONED MEDIUM ON THE LAG PHASE

(Age of inoculum, 7 days; average of four experiments)

MEDIUM	HOURS									
	0	2	4	6	12	14	24	36	48	72
Control	100	100	105	130	350	640	2,600	21,000	85,000	126,000
Conditioned (60-hr. centrifugate)	100	125	160	240	710	1,600	6,300	15,500	23,000	30,000

Mast and Pace (1938), after a long series of experiments, concluded that *Chilomonas paramecium* elaborated a substance (designated as X), which in low concentrations accelerates the growth of freshly inoculated chilomonads "from a vigorous culture" (p. 361) (optimum 1 part in 10,000,000) but inhibits growth or causes death when the concentration becomes high. They claim to have demonstrated the "Robertson effect," in that the rate of reproduction was higher when many chilomonads (up to 50) were inoculated into a given small volume (0.4 cc.) than when few (1 or more) were used. Furthermore, they show that as the volume of culture fluid is decreased the rate of reproduction of a single chilomonad rises. They believe that the elaborated substance is necessary for reproduction and that when the substance is low in the medium a certain amount of time is needed for its production. They, therefore, confirm the original hypothesis of Robertson under considerably more controlled conditions. They state that substance X is heat labile, being destroyed in proportion to the time of heating (100° C.). These conclusions are of great significance.

The accelerative effect which was demonstrated in the present study seems to compare favorably with the work on *Chilomonas*. It would be necessary only to assume that the so-called X-substance is elaborated more slowly in *Tetrahymena* and that it does not act in such high dilutions. Moreover, it has not been shown to be lethal in high concentrations, although it does lose its accelerative powers with age. Perhaps, if both the

Tetrahymena and the *Chilomonas* materials had been handled in the same manner, more points of similarity would have been noted.

It does not seem probable that a comparison can be made between the inhibitory effect of the conditioned filtrate and the inhibition noted by Mast and Pace, because of differences inherent in the techniques employed. Although, if one is to look upon the inhibitory effect of the filtrate as being due to elaborated substances, then it may be that the old *Tetrahymena* culture medium contains more and more of this substance, thereby masking the effect of a possible accelerative substance. The experiments of Mast and Pace, wherein they heated their *X*-containing medium for varying periods of time, seem to rule out direct comparison, however.

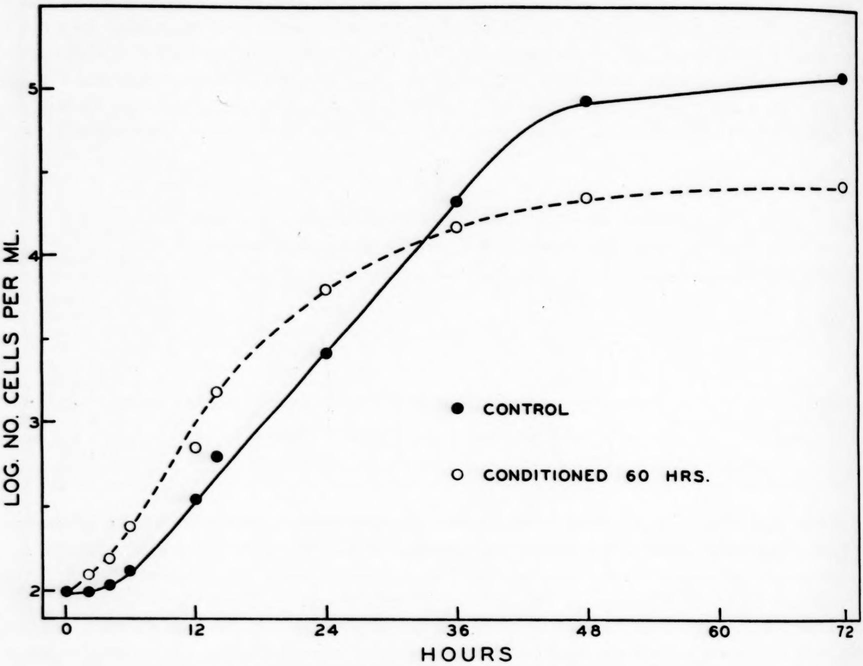

FIG. 6.—Diagrammatic representation of the data given in Table 7

A possible explanation of the phenomena reported here may be tentatively expressed as follows. In a growing culture of *Tetrahymena* two types of substances are being elaborated by the cells—one an accelerator which is produced at a high rate during the first few hours (up to 60) of growth, the other type detrimental and inhibiting to growth. Both types of substances are heat labile so that heat-treated conditioned medium is restored to the level of fresh broth (as far as early growth rate is concerned). The accelerator appears to dominate the activity of the growing cells because it exerts its effect in the presence of the inhibitor as in the centrifugate. Removal of the accelerative substance or substances seems to be brought about by filtration or adsorption, thereby allowing the inhibitor to become effective. It is evident that the accelerative substances are not removed by the filter because of their size (mechanical filtration) but by adsorption (see the experiment with Norit), but it is also indicated that they may be able to change their electrical charge as they appear to be adsorbed on electropositive plaster of Paris.

Growth inhibition due to filtrates from old cultures seems to be the rule in bacterial studies. Inhibitory substances have been shown to pass through a collodion membrane (Wheeler and Stuart, 1937) and collect in a sterile broth which was separated from a growing culture. There is the possibility that, could the previous population of a bacterial culture be removed by centrifugation rather than by filtration, then acceleration would be the result.

A more complete understanding of the substances involved during biological conditioning must await further investigation. Carbon dioxide tension, which is known at times to accelerate in high dilutions and depress in higher concentrations, might be a factor in the present study. There is some evidence against this interpretation, however. Untreated medium has a pH of 6.8. Medium conditioned for 60 hours has a pH of 5.8–6.0. This drop in pH value occurs only in the presence of the sugar, as plain proteose-peptone medium grows slightly alkaline with the advancing age of the culture. When conditioned medium is passed through a Seitz filter, the first 10–15 ml. collected has a pH of 6.8, showing that the acid-producing material is being taken out. This might be carbon dioxide as filtration brings about rather complete aeration. But subsequent collections from the filter show that the pH of the filtrate is dropping. After 100 or more ml. have passed through, the value is very near that of the centrifugate of the conditioned medium. This indicates that the filter is actually adsorbing the acid-forming substance and that eventually the filter pad becomes saturated. It is difficult to imagine the acid-forming substance as being CO_2, for aeration is just as complete at the end of a filtration as at the beginning. Of course, the shift in pH value during filtration may be due to the addition of some basic substance from the filter, but in either case the importance of CO_2 is minimized.

Recently Hall and Loefer (1940) reported the effects which they obtained by the use of old, uninoculated medium and old culture filtrate. Because of the rather obvious connection between their claims and the data reported here (in fact, the statement is made that I confirm the results given in their earlier abstract [Hall and Loefer, 1938]) it will be necessary to analyze their reports in some detail. The organism they used for their experiments is called by them *Colpidium campylum* but is the same strain of *Tetrahymena* which I used (Hetherington strain, see above).

Because of the method of handling their material I do not agree that my data (reported in abstract form [Kidder, 1939*b*]) confirms their results or their interpretations. The following points will suffice, I believe, to show that the conditions under which they worked could not have been expected to bring out data which could be used for comparisons with previous work or the data reported here.

They claim to have obtained evidence that the addition of a filtrate (Whatman No. 12 filter paper!) of an old culture (7 weeks–3 months) or the addition of sterile aged (3 months) medium to fresh medium increased both the rapidity of growth and the yield of their ciliates. Their basic medium was a casein peptone (Difco tryptone), and the efficacy of this medium can be judged by the control of their first experiment (Series II), in which, after 144 hours with an initial inoculum of 67 ciliates per ml., the yield was only 1,327 ciliates per ml. Compare this to the controls (unconditioned medium) given above when a more adequate medium is used (100,000–125,000 per ml. at 72 hours). Furthermore, there is a number of discrepancies in their report. In the first set of experiments their inoculum was from a 48-hour stock culture, and each tube received 1.0 cc. volumes of the inoculum. This inoculum resulted in an initial concentration of 67 per cc. In their second and third experiments each tube received only 0.5 cc. from a 48-hour culture, and yet they give the initial concentration of all tubes in these two experiments as 1,219 ciliates per cc. One can only conclude that they are getting remarkable inconsistencies in growth in their stock cultures. The control in the first experiment yielded only 1,327 ciliates per cc. at the end of 144 hours, which they consider as better than their Series V and VIII (controls), because the increase in the first case was twenty times the inoculum at the end of 6 days, while in the others it was less than eight times the inoculum. It is difficult to reconcile this statement with the easily demonstrated fact that no matter what the number of cells in the inoculum, provided it is less than the number reached at the end of logarithmic growth and provided the inoculated cells live, the yield is the same under the same conditions of medium and temperature. We have always been able to verify this fact; and it was perfectly demonstrated in the work of Phelps (1935), as it has been in innumerable cases with bacteria and yeasts.

Hall and Loefer were not using what I refer to here as biologically conditioned medium, because after aging, either in the presence or in the absence of ciliates, they subjected their experimental fluids to the temperatures and pressures of the autoclave rather than removing the previous population aseptically.

The demonstration of both acceleration of growth and inhibition of growth from the medium which has supported a previous population would seem to leave the way open for a revaluation of some of the contradictions in the literature regarding the actuality of the "Robertson effect." This is made difficult, however, by the fact that very few of the investigations dealing with this question were carried on under controlled conditions. We have no notion as to the role of the associated microörganisms in the reports; so it appears that, if this interesting question is to be settled, the early work will have to be repeated on sterile organisms. Even then the *modus operandi* will not be understood until we are in a position to make biochemical analyses of what may possibly prove to be elaborated enzymes or even more delicately arranged chemical materials. At present we can only point to the results and admit that the explanations are conjectures.

SUMMARY

1. When sterile *T. geleii* are grown in proteose-peptone–dextrose broth, they exhibit reproducible growth characteristics.

2. Young ciliates inoculated into fresh broth exhibit no lag phase but reproduce logarithmically until the negative acceleration phase is reached, when the division rate decreases.

3. Old ciliates inoculated into fresh broth exhibit a lag phase, but the maximum yield is the same as when the inoculum is young.

4. Medium which has supported a population of ciliates for up to 60 hours possesses the power either to accelerate or to inhibit the growth of a subsequent population. If the previous population is removed by centrifugation, acceleration results. If the pre-

vious population is removed by passing the fluid through a bacteriological filter (Seitz of Berkfeld), inhibition results.

5. The accelerating factor or substance decreases with the age of the previous culture after 60 hours, so that medium conditioned 4 weeks shows only slight but significant acceleration while medium conditioned 8 weeks shows no acceleration (as compared to fresh broth).

6. Medium conditioned 4 and 8 weeks and then filtered shows progressively more inhibition.

7. Centrifuged, 60-hour conditioned medium when treated with sterile charcoal (Norit) or plaster of Paris loses its accelerative ability and produces inhibition of growth.

8. All conditioning effects, except the lower maximum yield, are destroyed by heat and also lose their activity upon standing.

9. Centrifuged, 60-hour conditioned medium when inoculated with old ciliates diminishes or obliterates the lag phase.

10. It is suggested that two substances may be elaborated by the growing ciliates, one an accelerator which acts freely during the early growth phases but which is removed either by filtration or by adsorption. The other substance produces inhibition of growth, and when the accelerator is removed its full effect is noted.

LITERATURE CITED

Allee, W. C. 1931. Animal aggregations. Chicago: University of Chicago Press.

———. 1934. Recent studies in mass physiology. Biol. Rev., **9**:1–48.

Buchanan, R. E. 1918. Life phases in a bacterial culture. Jour. Infect. Dis., **23**:109–25.

Cutler, D. W., and Crump, L. M. 1923*a*. The rate of reproduction in artificial cultures of *Colpidium colpoda*. I. Biochem. Jour., **17**:174–86.

———. 1923*b*. The rate of reproduction in artificial cultures of *Colpidium colpoda*. II. *Ibid.*, pp. 875–86.

———. 1924. The rate of reproduction in artificial cultures of *Colpidium colpoda*. III. *Ibid.*, **18**:903–12.

———. 1925. The influence of washing on the reproductive rate of *Colpidium colpoda*. II. *Ibid.*, **19**: 450–53.

Furgason, W. H. 1940. The significant cytostomal pattern of the "*Glaucoma-Colpidium* group" and a proposed new genus and species, *Tetrahymena gelii*. Arch. f. Protistenk. Vol. **94**.

Hall, R. H. 1938. The oxygen-consumption of *Colpidium campylum*. Biol. Bull., **75**:395–408.

Hall, R. P., and Loefer, J. B. 1938. Effect of the addition of old culture medium on the growth of *Colpidium campylum*. Anat. Rec., **72**:50 (abstract).

———. 1940. Effects of culture filtrates and old medium on growth of the ciliate *Colpidium campylum*. Proc. Soc. Exper. Biol. and Med., **43**:128–33.

Kidder, G. W. 1939*a*. The effect of biologically conditioned medium on the growth of *Colpidium campylum*. Biol. Bull., **77**:297–98 (abstract).

———. 1939*b*. The effects of biologically conditioned medium on the growth rate and population yield of certain ciliated protozoa. Science, **90**:405–6 (abstract).

Kidder, G. W.; Lilly, D. M.; and Claff, C. L. 1940. Growth studies on ciliates. IV. The influences of food on the structure and growth of *Glaucoma vorax* sp. nov. Biol. Bull., **78**:9–23.

Kidder, G. W., and Stuart, C. A. 1939. Growth studies on ciliates. I. The role of bacteria in the growth and reproduction of Colpoda. Physiol. Zoöl., **12**:329–40.

Mast, S. O., and Pace, D. M. 1938. The effect of substances produced by *Chilomonas paramecium* on rate of reproduction. Physiol. Zoöl., **11**:359–82.

———. 1939. The size of the molecules in the growth substance produced by *Chilomonas paramecium*. Anat. Rec. Suppl., **75**:77 (abstract).

Park, T. 1939. Analytical population studies in relation to general ecology. Amer. Midland Naturalist, **21**:235–53.

Phelps, A. 1935. Growth of protozoa in pure culture. I. Effect upon the growth curve of the age of the inoculum and the amount of the inoculum. Jour. Exper. Zoöl., **70**:109–30.

ROBERTSON, T. B. 1921*a*. Experimental studies on cellular multiplication. I. The multiplication of isolated Infusoria. Biochem. Jour., **15**:595–611.

———. 1921*b*. Experimental studies on cellular multiplication. II. The influence of mutual contiguity upon reproductive rate in Infusoria. *Ibid.*, **15**:612–19.

———. 1922. Reproduction in cell communities. Jour. Physiol., **56**:404–12.

———. 1924*a*. The nature of the factors which determine the duration of the period of lag in cultures of Infusoria. Australian Jour. Exper. Biol. and Med. Sci., **1**:105–20.

———. 1924*b*. The influence of washing upon the multiplication of isolated Infusoria and upon the allelocatalytic effect in cultures initially containing two Infusoria. *Ibid.*, pp. 151–75.

———. 1924*c*. Allelocatalytic effect in cultures of *Colpidium* in hay infusion and in synthetic medium. Biochem. Jour., **18**:1240–47.

———. 1927. On some conditions affecting the viability of Infusoria and the occurrence of allelocatalysis therein. Australian Jour. Exper. Biol. and Med. Sci., **4**:1–24.

WHEELER, K., and STUART, C. A. 1937. Growth inhibition of *Echerichia coli*. Jour. Bact., **33**:525–31.

A FURTHER STUDY OF ENVIRONMENTAL FACTORS AFFECTING CYSTMENT IN WOODRUFFIA METABOLICA

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NORMALLY, *Woodruffia metabolica* forms resting or protective cysts in the absence of food (Johnson and Evans, 1939). Depending upon environmental conditions at the time of encystment, this ciliate will form one of two types of protective cysts, which are distinguishable both physiologically and morphologically (Johnson and Evans, 1940). We refer to them as "stable" and "unstable" cysts. Excystment of stable cysts occurs only upon the addition of some plant extract, while the excystment of unstable cysts can be induced by the mere addition of distilled water or by the abrupt lowering of the temperature. Morphologically, the unstable cysts are distinguished from the stable type by the possession of fluid vacuoles, larger protoplast, more diffuse cytoplasmic granules, less viscosity of the protoplasm, and generally a thinner ectocyst and mesocyst.

Other investigators have reported cysts of protozoa which can be induced to excyst without the addition of any organic substance. For example, Moore (1924) has reported the spontaneous excystment of *Spathidium.* Darby (1929) was able to cause excystment of *Stylonychia* by addition of fresh water to the cysts. Garnjobst (1937) induced excystment of *Euplotes taylori* and *Stylonethes sterkii* upon the addition of tap or distilled water. Kidder and Stuart (1939) regularly brought about excystment of *Colpoda* simply by adding distilled water. Taylor and Strickland (1938) have observed the spontaneous excystment of *C. duodenaria* under certain conditions in sterile inorganic medium. *Woodruffia,* which have been induced to encyst and excyst three or four successive times without feeding, have been seen to excyst spontaneously in inorganic medium upon numerous occasions; the sensitivity of these small cysts to slight changes of the environment is apparently intensified. A comparative study of the different protozoan cysts referred to above should be carried out in the same laboratory.

The occurrence of unstable cysts in *Woodruffia* was discovered quite by accident. During some tests in which encysting organisms were subjected to higher temperatures, we were surprised when the mature cysts in control dishes excysted merely with the addition of distilled water. It was also striking that the excystment time was only 50–75 minutes at room temperature, whereas the excystment time of stable cysts is 12–15 hours under the same conditions. Experiments were then devised to find the factors which caused the formation of unstable cysts, the results of which have already been reported (Johnson and Evans, 1940). In that study it was found that various factors such as high temperature, excessive crowding, starvation of food organisms, and moderately increased salt concentration of the medium were effectual to a small degree in stimulating the formation of unstable cysts. When these factors were combined, the stimulating effect was considerably increased. It was also found that a total salt concentration of 1.2 per cent (Osterhout's balanced salt one hundred times that generally used) would induce nearly 100 per cent formation of unstable cysts.

The purpose of the present investigation was to extend these studies on factors concerned in the formation of unstable cysts and to determine if possible whether several factors acting independently operate in producing the unstable cyst or whether there is some underlying single factor which causes the formation of such cysts.

MATERIALS AND METHODS

The *Woodruffia* used for this investigation were derived from one of our stock cultures. The methods used in maintaining stock cultures have been described in detail elsewhere (Johnson and Evans, 1940). *Paramecium multimicronucleata* was used as the food organism in both the stock cultures and the cultures of *Woodruffia* which were used as experimental animals.

A number of carefully washed paramecia were transferred to isolation cultures each containing a suspension of *Pseudomonas fluorescens*. The progeny of the culture exhibiting the highest reproductive rate were distributed among four 250-cc. Erlenmeyer flasks each containing balanced salt solution. To each was added three standard loops of *P. fluorescens* daily. These flask cultures were kept at 26° C. After 2–3 weeks the maximum population of paramecia was reached (450–500 per cubic centimeter); until this time none was used as food for *Woodruffia*. The paramecia of such cultures were similar in size and appearance. Whenever paramecia were pipetted out for *Woodruffia*, fresh balanced salt was added to the flask to maintain the original volume.

The bacteria were obtained from the Stanford department of bacteriology from the strain designated "U6" and grown on Difco nutrient agar. The bacteria used as food for the paramecia were always taken from 4-day-old plates.

Hundreds of paramecia were prepared for use daily by washing twice in balanced salt solution by centrifuging. These paramecia were transferred to Columbia watch dishes, to each of which was then added a few *Woodruffia*. An adequate source of experimental animals was thus available at any time.

As long as food is present, most of the *Woodruffia* are either engaged in division or have formed temporary or digestion cysts. The latter type resembles division cysts, but no division occurs. When the food has been depleted in a culture, all the *Woodruffia* become free swimming almost simultaneously for a short time. At this cultural phase, experimental animals were removed and washed, preparatory to encystment.

Balanced salt solution was prepared with triple Pyrex-glass–distilled water and C.P. salts (Merk.), and buffered at a pH of 6.9–7.0. Johnson (1933) has described the method for preparing the salt solution. The solution was filtered through a porcelain filter before use. *Woodruffia* to be used for experiments were always washed twice in the balanced salt solution by transferring with clean pipettes.

Columbia watch glasses were used as experimental dishes. Before use these dishes were cleaned thoroughly by scouring, then rinsed in distilled water, and dried with a clean towel. Experiments at 10° C. were carried out in a constant-temperature room with a fluctuation of not more than 0.2° C. Experiments at all higher temperatures were conducted in box incubators with a fluctuation of not more than 0.4° C. Petri dishes (diameter, 10 cm.) were used as moist chambers. At higher temperatures (30–37° C.) the Petri dishes were placed in large moist chambers for added insurance against evaporation.

In the following experiments *Woodruffia* experimental cultures were prepared in two ways. The method most generally used consisted in transferring 30 washed *Woodruffia* to an experimental dish containing 1 cc. of medium. In the other method hundreds of washed paramecia contained in 0.5 cc. of medium were placed in an experimental dish to which was added one washed *Woodruffia*. The latter type of culture will hereafter be referred to as a "completion culture." Completion cultures were prepared for tests at higher temperatures only (30–35° C.).

EXPERIMENTAL

It has already been demonstrated that a high percentage of unstable-cyst formation can be induced by increasing the concentration of salts in solution and that other factors such as moderately high temperatures, crowding, and starvation of food organisms contribute to a lesser degree in causing the production of such cysts. It would seem that the above conditions, which we have been able to produce in the laboratory with facility, would be considered as extreme when operating in nature, though possibly not infrequently encountered. Are unstable cysts formed under the influence of extreme conditions in general, or is there some underlying single cause?

I. HIGH TEMPERATURE

Earlier experiments on the effects of temperature on formation of unstable cysts have shown that there is a considerable increase in production of such cysts with increase in temperature when using completion cultures. In that study it was found that at 30° C. approximately 95 per cent of the cysts formed were unstable, and at 25° C. approximately 55 per cent. In an endeavor to repeat these results similar cultures were prepared using comparable food organisms. Precautions against evaporation were exercised. Table 1 shows the results. The formation of unstable cysts in the controls (25° C.) averages 62 per cent, and at 30°, 98 per cent. Completion cultures subjected to 35° C. show virtually 100 per cent formation of unstable cysts. At 35° C. the food organisms in the completion cultures had been consumed, and the *Woodruffia* were beginning the encystment process at the end of 1½ days; at 30° C. the time required was 2 days; and at 25° C., 3½ days. When the cysts in a given series were mature, generous samples were segregated, and the numbers of stable and unstable cysts counted; then the medium was removed, and distilled water added to test for activation.

TABLE 1

EFFECTS OF TEMPERATURE

(Completion cultures)

	Experimental						Control			
Series	1	2	3	4	5	6	1	2	3	4
No. of cultures	6	6	6	6	12	12	6	6	6	6
Temperature (° C.)	35	35	35	35	30	30	25	25	25	25
Percentage formation*	100	99+	99+	100	99	97	44	63	76	68
Percentage activation	100	100	100	100	100	100	85	87	90	81

* Percentage formation was determined by microscopical observation; percentage activation, by the excystment upon the addition of distilled water.

Table 2 shows the results of experiments conducted to determine the effect of high temperature on uncrowded *Woodruffia* (30 per cubic centimeter) removed from their source of food and washed. The different series of experimentals and controls were run in parallel. In not a single case was unstable-cyst formation observed in the controls (25° C.). At 34° C. a few are formed, and the percentage rapidly increases as the temperature rises to 37° C., which is very close to the upper temperature limit of free-swimming *Woodruffia*. There is an apparent correlation between the percentage of unstable cysts and the percentage of activation, as though the presence of many such cysts somehow facilitated their excystment.

The use of completion cultures augments the formation of unstable cysts for a number of possible reasons. Crowding at the time of encystment, semistarvation of food organisms, increase in concentration of solutes in the medium by accumulation of metabolites, and the undisturbed condition of the *Woodruffia* at the onset of encystment no doubt have their influence. It has been our experience that agitation of *Woodruffia* by sucking in and out of a micropipette and thus delaying encystment for 3–5 hours tends to prevent the formation of unstable cysts; this was the primary reason for the use of washed *Woodruffia* in uncrowded cultures.

TABLE 2

EFFECTS OF HIGH TEMPERATURE

A. EXPERIMENTAL

Series	1	2	3	4	5	6	7	8	9	10
No. of cultures	6	6	6	6	6	6	6	6	6	6
Temperature (° C.)	34	34	34	35	35	36	36	36.5	36.5	37
Percentage formation	4.7	7.6	2.7	10.5	19	35	47	66	76	91
Percentage activation	3.2	7	......	48	76	72	86	81	91	98

B. CONTROL

Series	1	2	3	4	5	6	7	8	9	10
No. of cultures	6	6	6	6	6	6	6	6	6	6
Temperature (° C.)	25	25	25	25	25	25	25	25	25	25
Percentage formation	0	0	0	0	0	0	0	0	0	0
Percentage activation	......	......	......	......	......	......	......	......	......	......

II. LOW TEMPERATURE

Before the existence of unstable cysts was known, free-swimming *Woodruffia* were exposed to low temperatures in attempting to induce encystment by this means. It was found that organisms subjected to 10° C. formed cysts nearly twice the size of those formed at higher temperatures. It was our desire to find whether such cysts were unstable.

Experimental cultures of 30 *Woodruffia* each were placed in a 10° C. constant-temperature room. This low temperature slowed the encystment process markedly, and for this reason the cultures were left 14 days before removing for observation and testing. Table 3 reveals that all experimental dishes contained cysts of the unstable type; however, it was observed that certain differences from unstable cysts formed under other conditions existed. For example, the cyst wall is several times thicker, as measurements have shown (see Table 4). Both stable and unstable cysts formed at 10° C. show this same character of the wall. Another difference is the fact that unstable cysts formed at 10° C. require 3–4 hours for excystment, whereas such cysts produced under other conditions require but one-third the time. Excystment of 10° unstable cysts seldom occurs by substitution of distilled water for the encystment medium alone; but if the cysts in distilled water are transferred to a glass slide and a cover slip placed over them, good excystment usually takes place. No study of this problem has been made; however, pressure of the

TABLE 3

EFFECTS OF LOW TEMPERATURE (10° C.)

Series	Number of Trials	Average Percentage Formation	Average Percentage Death	Average Percentage Activation	Series	Number of Trials	Average Percentage Formation	Average Percentage Death	Average Percentage Activation
1........	3	24	19.0	....	7........	4	82	9.0	60
1*a**......	1	0	0	....	8........	4	83	15.0	10
2........	2	75	10.5	....	9........	4	58	16.0	0?
3........	4	90	7.0	75	10........	4	55	18.0	20
4........	4	56	21.0	75	11........	4	47	30.0	15
5........	4	77	23.0	75	12........	3	34	16.0	75
6........	4	82	15.0	60	13........	3	74	13.0	80

* Control at 25° C. Controls for the remainder of the series are not recorded, as the results were identical.

TABLE 4

MEASUREMENT OF CYSTS

ROOM-TEMPERATURE CYSTS		10° CYSTS	
Protoplast	Protoplast and Wall	Protoplast	Protoplast and Wall
50............	77	95*	153
58............	84	87	175
66............	87	87	168
54............	80	90*	190
80............	102	77	160
58............	77	87	153
67............	80	109*	146
46............	69	131*	175
58............	77	75*	138
77............	109	80	146
		102*	146
		109*	204
		131*	168
		116*	146
		116*	175
		109	182
		138*	197
		80	160
		95	131
		109*	138

* Unstable cysts.

cover slip may be a direct cause of excystment. It could not be induced by raising the temperature from 10° C. In all the 10° C. cultures some death occurred as recorded in the table. Controls at 25° C. showed neither unstable-cyst formation nor death.

III. HYDROGEN ION CONCENTRATION

Three sets of buffer mixtures were used, namely, acid potassium phthalate, pH 5.0–6.0; acid potassium phosphate, pH 6.0–8.0; boric acid–potassium chloride, pH 8.0–9.5 (Clark, 1925). The method for preparing the mixtures has been described elsewhere (Johnson and Evans, 1939). Determinations of H-ion concentrations were made with LaMotte's colorimetric standards.

Experimental animals were washed twice in the solution to be tested, and from the second wash 30 organisms were transferred with as little fluid as possible to the experimental dish containing 1 cc. of medium. Table 5 shows that in the vicinity of the acid-death point the formation of unstable cysts is greatest and that only within the narrow range of pH 5.6–6.0 were any such cysts formed. Alteration of the pH to neutrality was not effective in activating unstable cysts; addition of distilled water was necessary. Addition of organic medium (alfalfa extract) induced the excystment of stable cysts. Hydrogen ion concentrations less than a pH of 9.5 were not tested.

TABLE 5

EFFECTS OF H-ION CONCENTRATION ON FORMATION OF UNSTABLE CYSTS

pH	Trials	Average Percentage Formation	pH	Trials	Average Percentage Formation
5.0	4	Death in 1 hour	6.6	4	0
5.2	4	Death in 2 hours	7.0	4	0
5.4	4	Death in 24 hours	7.5	4	0
5.6	9	78	8.0	4	0
5.8	9	50	8.5	4	0
6.0	9	10	9.0	4	0
6.2	9	0.5*	9.5	4	0
6.4	4	0			

* Formation in only one dish.

IV. LOW OXYGEN TENSION

The fact that crowding, increasing the salt concentration, and increasing the temperature tend to facilitate the formation of unstable cysts and that a mere lowering of the temperature will induce them to excyst suggests that changes in oxygen tension may play a role both in the formation and in the activation of such cysts. If this relationship exists, only slight changes in oxygen tension would suffice in effecting the changes under consideration.

Experiments were devised to test this matter. An airtight system whereby *Woodruffia* could be maintained indefinitely in an atmosphere of nitrogen (99.7 per cent pure) or mixtures of air and nitrogen was set up. Two holes were bored through the lid of a Petri dish (diameter 10 cm.) on opposite sides, and an S-shaped glass tube sealed to each with De Khotinsky. The inlet to the Petri dish led from a liter Erlenmeyer flask filled with distilled water in which extended to the same depth two glass tubes (5 mm. inside bore). One of these admitted nitrogen (Linde high-test nitrogen), and the other, air under compression. By manipulation of valves the stream of bubbles of air and nitrogen could be regulated to give the desired proportions. The gas then passed to the chamber containing the experimental animals and thence to a 250-cc. Erlenmeyer flask where the escaping gas bubbled through water surfaced with mineral oil. With this setup it was possible to maintain the small cultures without any apparent decrease in volume of the medium over a period of 3 weeks.

At the beginning of an experiment a Columbia dish containing 40 washed experimental animals was placed in the Petri dish, and the two halves sealed together with hard paraffin wax. This assembly was put on the stage of a dissecting microscope. All joints of the system were also coated with the wax. Observations were made several times daily.

The first few trials were made by passing nitrogen unmixed with air through rapidly. The *Woodruffia* were dead within 3 hours in all these trials. In the following trials mixtures of air and nitrogen were used in varying proportions. When 1 bubble of air to 1 bubble of nitrogen was passed through the experimental chamber, the *Woodruffia* formed stable cysts in the normal time. The same was true when 1 bubble of air to 3 of nitrogen was tried. When 1 of air to 25 of nitrogen was tried, some of the organisms came to the surface of the encystment medium to encyst; but all formed stable cysts. All the organisms came to the surface to encyst when 1 bubble of air to 50 of nitrogen was tried; all cysts were stable. The same happened with 1 of air to 100 of nitrogen. In all the above experiments each *Woodruffia* divided once before encysting, and the encystment time was normal. When 1 bubble of air to 200 of nitrogen was passed through the experimental chamber, the encystment time was delayed approximately $1\frac{1}{2}$ days, and the number of cysts totaled only 50; still all were stable. It has been found by Ephrussi, Chevillard, Mayer, and Plantefol (1929) that cell division of chick-embryo fibroblasts cultured *in vitro* was arrested at reduced oxygen tensions (7 mm. Hg.).

In the following trials the stream of nitrogen gas was slow but continuous (20–25 bubbles per minute) and the amount of air was limited to a few bubbles a day (no more than 30). When 30 bubbles of air were allowed to pass through, 1 week was required to complete encystment. The number of cysts amounted to 42, of which 20 were dead. Half of the living were unstable, and 30 per cent of these were activated by distilled water; excystment time was delayed as in the case of cysts formed at 10° C. In another trial using the same proportion of gases similar results were obtained. At the end of the experiment 39 cysts were present, 5 of which were dead; 54 per cent were unstable with 85 per cent activation. Four of the cysts had apparently activated without breaking through the cyst wall before completion of the experiment. All 4 had re-encysted to form the stable type. No activation could be induced by increasing the oxygen tension. All stable cysts formed under these conditions were viable when tested with the usual organic excystment medium.

When the amount of air was reduced to 20 bubbles per day, only 12 cysts remained alive, 10 of which were unstable. When the amount of air was reduced to 10 bubbles per day, all organisms were dead within 8 days.

It is obvious from the foregoing experiments that a much greater reduction in oxygen tension is required for the formation of unstable cysts than results from the crowding of organisms, the increasing of the salt content, or the raising of the temperature. Only a rough approximation of the oxygen tension is possible, but it can be said that unstable cysts were not formed until the percentage of oxygen in the air surrounding the culture lay at least below 0.1 of 1 per cent.

V. LOW OXYGEN TENSION WITH FOOD

In an earlier study (Johnson and Evans, 1939) it was found that of a wide variety of experimental conditions (quantity of food, pH, temperature, salt concentration, slow evaporation, crowding, metabolites, quality of food) only lack of food would induce the formation of protective cysts in *Woodruffia*. In these experiments the oxygen content had not been varied, and all the cultures were small in volume with a large surface area exposed. In the present work an attempt was made to control the oxygen tension by the method employed above. Each experimental dish at the beginning of an experiment contained 10 *Woodruffia* and hundreds of paramecia in 0.5 cc. of medium.

The first trial was made by passing 1 bubble of air to 10 bubbles of nitrogen through the chamber. During the first 3 days the fission rate amounted to about 1.3, whereas at that temperature a fission rate of at least 2.0 is to be expected. On the fifth day encystment of the *Woodruffia* began, even though food organisms were still numerous. Encystment was complete 2 days later; the number of food organisms had been reduced to one-tenth the number present at the beginning. There were about 1,500 cysts, 10 per cent of these being unstable as determined both by observation and by testing for excystment. The similarity of this culture to a completion culture probably accounts for the presence of unstable cysts. When 1 bubble of air to 20 of nitrogen was passed through the system, similar results were obtained; however, encystment began 1 day sooner. The fission rate amounted to 1.0. The number of food organisms remaining at the end of the cultural period was considerably more than in the first experiment. There were about 1,000 cysts, 3 per cent of which were unstable. In this case the similarity to a completion culture was lessened probably by the reduced oxygen tension.

When 1 bubble of air to 30 of nitrogen was used, the fission rate for the first 3 days fell to less then 0.45, and by the fifth day all had encysted to form 48 stable cysts. Any decrease in number of food organisms was not noticeable. This experiment was repeated with the formation of 65 stable cysts.

Then trials using 1 bubble of air to 60 of nitrogen were carried out. In the two tests tried, 19 and 22 cysts were formed respectively. Encystment commenced on the third day. In the first test 5 unstable and 4 dead cysts were found; in the second, all cysts were stable, but there was no death.

In the first trial using 1 bubble of air to 90 bubbles of nitrogen a leak appeared in the system on the third day, but during that time there was no division of the *Woodruffia* and no taking of food. The test was repeated. On the sixth day encystment began and on the eleventh day was completed. At the end of this period there were 12 cysts (indicating that only 2 of the 10 organisms divided before encystment), 5 of which were dead and 4 of the remaining were unstable. The three stable cysts were viable when tested with organic excystment medium. The duration of individual experiments varied between 7 and 20 days.

VI. AGING OF UNSTABLE CYSTS

A series of completion cultures was started at 30° C. to test the effects of age on viability. For the first 2 months, weekly tests were made on viability, the results of which have already been reported (Johnson and Evans, 1940). At the end of 2 months no change in viability had occurred. Thereafter, monthly tests were made, and at the end of 6 months the unstable cysts were still 100 per cent viable. It was noted, however, that with the passage of time the fluid vacuoles had increased both in number and in size. In all probability the end-result of this increased vacuolation will be the death of these encysted organisms.

DISCUSSION

According to the results obtained from the foregoing experiments, formation of unstable cysts seems to depend upon the occurrence of extreme conditions in general at the time of encystment. The existence of a single basic factor as the causal agent in the formation of such cysts appears to be unlikely. Since crowding, high temperature, and increased salt concentration facilitated unstable cyst formation, it was suggested that oxygen tension might be the single factor concerned; however, the oxygen tension required for the production of unstable cysts is so low that any such relationship does not appear likely. Another evidence against such a relationship is the fact that unstable cysts form at low temperatures. Among the various environmental conditions tested it was found that, as one approached the lethal point and as the percentage of deaths increased, the percentage of unstable cysts increased. Correlated with this was the increasingly prolonged encystment time, especially at low temperatures.

The finding here that decreased oxygen, as well as depletion of food, will induce encystment of *W. metabolica* even though ample food is present is in agreement with other workers. Rhumbler (1888) concludes that lack of air is a cause of encystment in *Colpoda*, though he describes no critical test. Adolph (1929) found that when the oxygen supply of *Colpoda* was suddenly removed, the organisms died; but when the removal was slower and not so complete some of the organisms formed permanent cysts, whereas none was formed in the controls. It was found by Taylor and Strickland (1938) that *Colpoda* are killed by lack of oxygen; however, it is their opinion that, if cyst formation can be induced by decreasing the oxygen tension, the direct factor is the inadequate supply of energy which is obtained from the food under these conditions. This situation may be true in the case of *Woodruffia*.

Amberson (1928) reports that the oxygen uptake of *Paramecium* is constant over a wide range (50–200 mm. Hg.) and that at 11 mm. Hg. the oxygen uptake is 80 per cent of that at atmospheric pressure. According to our experience, no effect of low oxygen tension was exhibited in *Woodruffia* until the partial pressure of the oxygen had been lowered to about 15 mm. Hg. As the pressure fell, the effect was augmented. Zoethout (1899) assumed that harmful oxidizable substances accumulate in the cell when deprived of oxygen. Such a situation might render the paramecia unfit as food, introducing, as a consequence, a condition comparable to absence or scarcity of food as far as the *Woodruffia* are concerned.

The lowest oxygen tensions seemed to have little or no apparent effect on the paramecia. Pütter (1905) found that paramecia remained living for as long as 10 days in some cases in the complete absence of oxygen. *Paramecium* has been found to inhabit the profundal bottom regions in lakes during stagnation periods of summer and winter (Welch, 1935). Here, according to analyses, no oxygen can be detected. Fauré-Frémiet, Léon, Mayer, and Plantefol (1929), by reducing the oxygen content by means of evacuation and also by means of reducing agents, found that *Paramecium* could survive up to

24 hours at 25° C. Kitching (1939) has reported that *Paramecium* will survive for 12 hours in anaerobic conditions produced by substituting especially purified hydrogen or nitrogen for atmospheric air. During this time decreasing activity and blistering was exhibited by the organisms. The organisms maintained a normal appearance at pressures as low as 1 mm. Hg.; death resulted at a partial pressure of oxygen of 0.2 mm. Hg.

The formation of permanent cysts in the presence of abundant food has been observed in one other situation. This phenomenon seemed to be associated with the use of old hay infusion as culture medium for *Woodruffia*. In these cultures the bottom of the dishes became matted with filamentous bacteria and fungi with other forms of microörganisms caught in the meshes. With such relatively enormous amounts of life in the small cultures it may be that the oxygen tension was lowered to a level insufficient to maintain the free-swimming *Woodruffia*. It was found that when the medium was filtered no such encystment occurred.

The true situation regarding the encystment of *Woodruffia* at low oxygen tensions when ample food is available cannot at present be certainly ascertained; however, three possibilities present themselves: (1) the effect on the *Woodruffia* may be direct, (2) the paramecia may not satisfy the energy requirements of the *Woodruffia*, and (3) the accumulation of harmful oxidizable substances in the food organisms may render them unfit as food. It is possible that all three factors may be operating simultaneously. However, the fact that the *Woodruffia* at the lowest oxygen tension did not remove enough of the food organisms to make any noticeable difference in their numbers favors the view either that there is a direct effect on the *Woodruffia* or that under these conditions the food organisms were so changed that they were no longer acceptable as food. In control cultures the same number of *Woodruffia* consume all the food organisms in the same period of time.

Cysts of different protozoa are known to remain viable for long periods of time. Stable cysts of *Woodruffia* (both wet and dry) were found to be viable after more than a year's time. Dawson and Hewitt (1931) report that dried cysts of *Colpoda* were viable after a period of 5 years and 4 months. Taylor in this laboratory has kept dried cysts of *Colpoda* for even longer periods, finding some loss of viability. Beers (1937) kept cysts of *Didinium* for 10 years with a gradual loss of viability. A good review of the literature is given in his paper.

Unstable cysts of *Woodruffia* cannot be dried without fatal results, so, for tests of aging, the cysts were maintained in the wet condition. Over the 6-month period no decrease in viability was observed. It was noted, however, that with the passage of time the fluid vacuoles increased considerably both in number and in volume. It seems probable that the source of energy necessary for the cytoplasmic movement which occurs in all unstable cysts is acquired by contributions from the substance of the body and that the diminution in the cytoplasmic volume is compensated by absorption of water. This condition would ultimately end in death of the cysts; and, according to an estimate of the cytoplasmic displacement, the life of the unstable cysts would be limited to but a few more months at most.

The viability of stable cysts decreases at a much slower rate. The metabolic activity transpiring in stable cysts appears to be slower than that in unstable cysts as adduced by the difference of movement between the two types. The situation in stable cysts seems to compare closely to that in cysts of *Colpoda* where the viability slowly falls.

SUMMARY

When free-swimming *Woodruffia* in absence of food were subjected to extremes of temperature (both high and low), high concentrations of H-ions, and low oxygen tensions, the formation of unstable cysts was induced. Under these extreme conditions a correlation between percentage of formation of such cysts and approach to the lethal point was noted. The cause of the production of unstable cysts could not be resolved to some single fundamental factor. The effect of low oxygen tension on feeding *Woodruffia* was tested and it was determined that as the oxygen tension fell the tendency to encyst increased, even though ample food was present. The direct cause for this phenomenon was not ascertained. The longevity of unstable cysts of *Woodruffia* was extended to 6 months with no decrease in viability.

LITERATURE CITED

ADOLPH, EDWARD F. 1929. The regulation of adult body size in the protozoan *Colpoda*. Jour. Exper. Zoöl., **53**:269–311.

AMBERSON, W. R. 1928. The influence of oxygen tension upon the respiration of unicellular organisms. Biol. Bull., **55**:79–91.

BEERS, C. DALE. 1937. The viability of ten-year-old *Didinium* cysts (Infusoria). Amer. Nat., **71**:521–25.

CLARK, W. M. 1925. The determination of hydrogen ions. Baltimore: Williams & Wilkins.

DARBY, H. H. 1929. The effect of the hydrogen-ion concentration on the sequence of protozoan forms. Arch. f. Protistenk., **65**:1–37.

DAWSON, J. A., and HEWITT, D. C. 1931. Longevity of encysted *Colpoda*. Amer. Nat., **65**:181–86.

EPHRUSSI, B.; CHEVILLARD, L.; MAYER, A.; PLANTEFOL, L. 1929. Recherches sur le besoin d'oxygène libre. II. L'oxygène libre et les cultures de tissus. Ann. physiol. et physicochim. biol., **5**:642–58.

FAURÉ-FRÉMIET, E.; LÉON, C.; MAYER, A.; and PLANTEFOL, L. 1929. Recherches sur le besoin d'oxygène libre. I. L'oxygène et les mouvements des paramécies. Ann. physiol. et physicochim. biol., **5**:633–41.

GARNJOBST, LAURA. 1937. A comparative study of protoplasmic reorganization in two hypotrichous ciliates, *Stylonethes sterkii* and *Euplotes taylori*, with special reference to cystment. Arch. f. Protistenk., **89**:317–81.

JOHNSON, W. H. 1933. Effects of population density on the rate of reproduction in *Oxytricha*. Physiol. Zoöl., **6**:22–54.

JOHNSON, W. H., and EVANS, F. R. 1939. A study of encystment in the ciliate *Woodruffia metabolica*. Arch. f. Protistenk., **92**:91–116.

———. 1940. Environmental factors affecting cystment in *Woodruffia metabolica*. Physiol. Zoöl., **13**:102–21.

KIDDER, G. W., and STUART, C. A. 1939. Growth studies on ciliates. I. The role of bacteria in the growth and reproduction of *Colpoda*. Physiol. Zoöl., **12**:329–40.

KITCHING, J. A. 1939. The effects of lack of oxygen and of low oxygen tensions on *Paramecium*. Biol. Bull., **77**:339–53.

MOORE, E. L. 1924. Endomixis and encystment in *Spathidium*. Jour. Exper. Zoöl., **39**:317–37.

PÜTTER, AUGUST. 1905. Die Atmung der Protozoen. Zeitschr. f. allg. Physiol., **5**:566–612.

RHUMBLER, LUDWIG. 1888. Die verschiedenen Cystenbildungen und die Entwicklungsgeschichte der holotrichen Infusoriengattung *Colpoda*. Zeitschr. f. wissensch. Zool., **46**:549–601.

TAYLOR, C. V., and STRICKLAND, A. G. R. 1938. Reactions of *Colpoda duodenaria* to environmental factors. I. Some factors influencing growth and encystment. Arch. f. Protistenk., **90**:396–409.

WELCH, PAUL S. 1935. Limnology. New York: McGraw-Hill Co. P. 170.

ZOETHOUT, W. D. 1899. On some analogies between the physiological effects of high temperature, lack of oxygen, and certain poisons. Amer. Jour. Physiol., **2**:220–42.